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Management of Chronic Idiopathic Thrombocytopenic Purpura in Children, with Particular Reference to Immunosuppressive Therapy[1]

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Ample evidence for the role of immunoallergic mechanisms in the aetiology of idiopathic thrombocytopenic purpura (ITP) has been presented [1 4 15 16, 22, 23 24]. The finding that purine-antagonists (6-mercaptopurine, thioguanine, and azathioprine) are capable of suppressing immune responses has led many authors to use these agents in the treatment of ITP [3, 7 20 31 34 36, 47 48]. However the natural history of untreated ITP has to be taken into account: approximately 80% of the affected children undergo spontaneous remission within 1 to 3 months [37 41 42]. Even among those who become chronic, spontaneous remissions as well as exacerbations can occur but complete remissions are rare. Because of this variegated and unpredictable course of ITP the critical evaluation of therapeutic results is particularly difficult. In this paper our experiences with azathioprine (Imuran) in the management of 4 patients with chronic idiopathic thrombocytopenic purpura who failed to show a spontaneous remission are described. Published reports on the effects of an immunosuppressive agent in the same condition are summarised in table I.

Materials and Methods

Four patients were selected under close observation for a period of between 9 months and 2 years, they showed only transient or no response to large doses of Prednisone but relapsed when this was reduced to below our standard initiating dose of 60 mg/m² day. All of them were fully immunized against diphtheria, tetanus, pertussis and poliomyelitis, or given one booster dose prior to the start of the immunosuppressive therapy as described earlier [27]. Since the effect of immunosuppressive agents may not become apparent for

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Table I Results of treatment with Imuran in thrombocytopenic purpura from various authors

Authors	Children		Adults	
	No. of patients	No. improved	No. of patients	No. improved
Bocksonell *et al.*, 1966 [7]	1	1	6	5
Gerhartz, 1967 [20]			1	1
Mariro *et al.*, 1966 [31]	6	2		
Mozschlin, 1966 [36]	1	1		
Schman, 1967 [47]	1	1	7	6
Swanson, 1967 [48]			2	2
Total	9	5	16	14

everal weeks, all patients were treated initially with combination of Prednisone (60 mg/m²/day) and Imuran (3–5 mg/kg/day) the former being gradually reduced after 4 to 8 weeks whereas the latter was maintained for at least 6 months. Regular clinical as well as haematological evaluations were carried out first in the hospital and subsequently in the Out-patients Department.

Routine haematologic methods were used. Leucocytes were counted with the Coulter Counter (Model D); platelet counts were made directly by enumerating them in counting chamber with phasecontrast microscope bleeding time was determined according to Bürker and coagulation time according to Duke. Coagulation studies were carried out according to various authors fibrinogen [10], prothrombin [29], factor V [28], factor VII [29], factor VIII and IX [19], factor X [2]. Immunoglobulins were assayed by immunodiffusion methods using monospecific antisera (one-dimensional procedure according to Oudin for IgG, two-dimensional modification of the Ouchterlony method for IgM and IgA as described earlier [26]) β 1C/A globulin was estimated qualitatively in an immunoelectrophoretic micromethod according to Scheidegger and semiquantitatively in two-dimensional immunodiffusion method using monospecific antiserum (Dutch Red-Cross, Amsterdam) CRP with a monospecific antiserum (Schieffelin Co.) haptoglobin according to Jayle [26]. The platelet adhesiveness was estimated according to the method described by Salzman [40]

Case Histories

Case 1 Sch. K. (4246/65) male, born in 1938, was said to have recurrent spontaneous bleeding manifestations since 1960. He was first admitted to our hospital in 1965 with history of an upper respiratory tract infection followed 2 days later by severe epistaxis and generalized purpura. There was no history of use of drugs or other known toxic substances. On examination he had numerous petechiae and ecchymoses of various sizes and positive tourniquet test but no hepatosplenomegaly nor lymphadenopathy. Investigations showed Hb 4.9 g%, retics 0.4%, thrombocytes 15,000/mm³ and leucocytes 8,000/mm³ with a shift to the left prolonged bleeding time and no clot retraction after 1 h at 37° C. Coagulation study LE- and direct antiglobulin tests, C-reactive protein, ASL titer and blood culture were either normal or negative. Bone marrow aspiration revealed an active marrow with normal cellular distribution and numerous megakaryocytes.

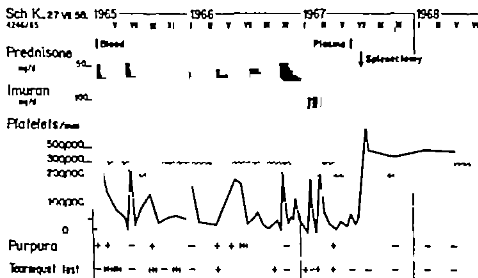


Fig 1 Case 1 Only high doses of Prednisone gave temporary increase in platelet counts. Imuran failed to achieve clinical remission over 7-months period on 2 occasions transient rise in platelet counts occurred following cessation of the Imuran therapy Fresh plasma transfusion did not bring the platelets to normal levels. Splenectomy ultimately led to clinical as well as haematological remission.

Thrombocytopenic purpura was diagnosed and treatment with blood transfusion on admission and 2 days later with Prednisone 55 mg daily was started (fig 1) Within one week the platelets rose to 991,000/mm³ but they gradually fell to 214,000/mm³ as Prednisone was tapered off to 20 mg, and to below 100,000/mm³ when this was further reduced to 10 mg daily Since discharge he has been regularly seen at our Out-patients Department and on 3 occasions he had to be readmitted on account of low platelet counts, associated with bleeding symptoms while on maintenance dose of 10–15 mg Prednisone daily During the 18 months follow-up from March 1965 to September 1966, it became obvious that the child responded only to high doses of Prednisone and spontaneous remission was unlikely Furthermore, side-effects of long term Prednisone therapy such retardation of growth (fig 2) obesity and radiological evidence of osteoporosis were observed.

On October 27 1966, in addition to the high dose of Prednisone, he was put on Imuran 125 mg daily (5 mg/kg/day) Prednisone was gradually reduced and completely discontinued within 8 weeks whereas Imuran was maintained at 125 mg daily for 7 months. He appeared to respond to the Prednisone-Imuran therapy t first, with rise of platelet count to 221,000/mm³ after one week treatment but soon the platelets fluctuated between 4,000 and 50,000/mm³ with recurrence of generalised ecchymoses and petechiae as well as positive tourniquet test on many occasions. His tolerance to the immunosuppressive agent was rather poor because of continuous nausea and vomiting since mid-January 1967 Imuran was discontinued for week in early February 1967 During this period, transient rise in platelet count up to 192,000/mm³ occurred unexpectedly (fig 1) One month later he developed scleral jaundice. There was slightly raised total serum bilirubin of 2 mg%, raised SGOT of 27.5 IU and slightly raised SGPT of 12.0 IU as well as positive uro-

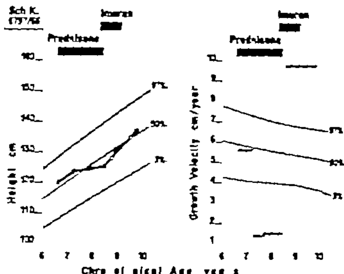


Fig. — Case 1 ( ) Effect of long-term Prednisone on growth. (b) Prior to the Prednisone therapy the growth rate is on the 50th percentile. When started on Prednisone there is striking decrease in growth rate to below 3rd percentile. After withdrawal of Prednisone there is 'catch-up' growth and his growth-rate increases to over the 97th percentile. The growth rate-chart for boys is taken from TANNER *et al.* (Arch. Dis. Childh. 1966).

bilinogen but negative bilirubin in the urine. α2-haptoglobin was normal. The presence of β_{1C}-globulin and absence of conversion to β_{1A}-globulin was thought to be against an immunological process with activation and consumption of complement. WBC was 3,000 and platelets 4,000/mm³. Imuran was therefore discontinued for a fortnight. Towards the end of this period, the platelet count had risen to 220,000/mm³. The increase in platelet count was nevertheless short-lived. Two weeks after re-introduction of Imuran therapy the platelet counts dropped to below 70,000/mm³ and remained low without leucopenia, for over two months when the Imuran was finally discontinued on June 1, 1967. After cessation of Imuran therapy thrombocytopenia remained unchanged but pruritus which was trouble some during Imuran therapy subsided. On June 13, 1967 a transfusion of fresh plasma 10 ml/kg was tried without satisfactory result. Subsequently splenectomy was carried out on July 1., 1967 with marked improvement in the platelet counts which remained unchanged for one year at levels between 300,000 to 500,000 mm³ (fig. 1). Observations made on the effects of Imuran on immunoglobulins and platelet adhesiveness are summarized in tables *II* and *III* respectively.

Summary: This child was said to have recurrent haemorrhagic diathesis since the age of years and ITP was diagnosed when he was 7 years old. He responded only to high doses of Prednisone and after approximately 2 years observation, it was concluded that spontaneous remission was unlikely. Side-effects of long term Prednisone therapy especially growth retardation were observed. Treatment with Imuran (3 mg/kg/day) for 7 months failed to achieve a remission. Ultimately splenectomy was carried out and the platelet counts following the operation have been maintained at levels above 300,000/mm³.

Case — R. F. (0328/66) male born in 1953, was known to have frequent bleeding episodes since 1963 and required hospitalizations in summer 1963 and in January 1963 in

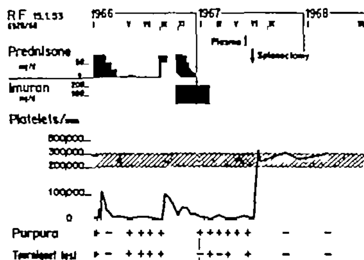


Fig. 3. Case 2. The patient failed to respond to Prednisone and Imuran therapy as well as fresh plasma transfusion. Following splenectomy however his platelet counts have been maintained at levels around 300,000/mm³

Italy. More detailed informations were however not obtainable. I January 1966 he was admitted to our hospital with two months' history of recurrent epistaxis, gingival bleedings, and purpura. Based on the history physical findings, blood picture with platelet count of 14,000/mm³ and bone marrow examinations, diagnosis of ITP was made. Platelet antibodies were however not detected. H responded temporarily to Prednisone 80 mg daily but relapsed following the reduction of Prednisone to 50 mg daily. After 10 months constant observation at our Out-patients Department, he had not responded to adequate Prednisone and had failed to show spontaneous remission.

On October 22, 1966, treatment with Prednisone and Imuran 200 mg daily (4 mg/kg/day) was started, but in vain. Apart from pruritus he tolerated the immunosuppressive agent well until in early February 1967 when he developed slight jaundice with serum bilirubin of 3.5 mg% and raised SGOT and SGPT of 29 and 22 IU respectively. Imuran therapy was therefore discontinued after 4 months on February 13, 1967. The pruritus which was troublesome during Imuran therapy ceased when the immunosuppressive agent was suspended. Fresh plasma transfusion was given on June 14, 1967 without effect. On July 7 1967 splenectomy was performed and the rise in platelet count following surgery was excellent, the number of platelets being kept at levels between 300,000 and 400,000/mm³ (fig. 3)

Case 3. O. C. (1027/64) male born in 1960, was admitted in March 1964 with history of recurrent haemorrhagic diathesis since the age of 15 months. There was no relevant family or personal history and no exposure to drugs or chemical agents was known. The significant findings consisted only in generalised petechiae and ecchymoses as well as positive tourniquet test. Thrombocytes 22,000/mm³ bleeding time prolonged, and clot retraction after one hour at 37°C poor. Bone marrow normal with adequate megakaryocytes. He was treated with Prednisone 45 mg per day initially with transient response but later during the 2 years' follow-up period remissions and exacerbations recurred, even

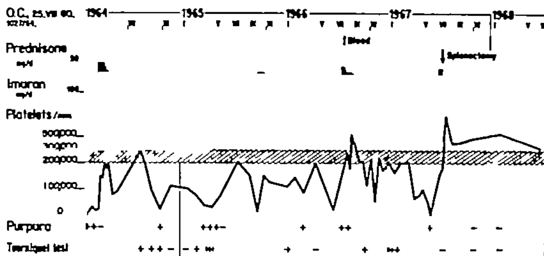


Fig 4 Case 3 Neither Prednisone nor Imuran therapy gave rise to satisfactory clinical remission. For the first 8 months he appeared to respond to Imuran but during the last 2 months he relapsed. Splenectomy has finally brought the platelets to normal levels.

when he was put on maintenance doses of Prednisone 5–10 mg daily The remissions were neither complete nor lasting

On August 13 1966, Prednisone and Imuran 100 mg daily (4 mg/kg/day) were begun, the former being gradually reduced after 4 weeks, whereas the latter was maintained for 10 months. The platelet counts fluctuated between 50,000 and 250,000/mm³ but during the last 2 months of Imuran therapy they maintained at levels between 5,000 and 75,000/mm³ At the end of May 1967 Imuran was discontinued. Splenectomy was carried out on July 14 1967 with satisfactory result; the platelet counts following operation have remained at levels above 300,000/mm³ (fig 4)

Case 4 S. U (8331/66) female born in 1961 presented with history of sudden onset of melaena and purpura fortnight prior to her admission in October 1964 Physical examination revealed numerous purpuric spots, mainly on the lower extremities but no other abnormal findings otherwise. Thrombocytes 4,000/mm³ bleeding time prolonged, and clot retraction was nil after one hour at 37°C. Coagulation study and repeated LE-tests were either normal or negative. Bone marrow was normal with numerous megakaryocytes. Prednisone 40 mg daily was begun with gradual increase in platelet count to 250,000/mm³ after 4 weeks of treatment but then fell to 10,000/mm³ on reduction of the Prednisone to 10 mg daily During the period of observation from November 1964 to October 1966 she had on 3 occasions to be treated with large doses of Prednisone for 6–8 weeks with temporary response however this resulted in transient glycosuria, facial rounding, moderat degree of hirsutism, and obesity

On October 19, 1966, Imuran 100 mg daily (5 mg/kg/day) in addition to Prednisone was prescribed, the latter being gradually tapered off. There was transient rise of platelet count to 300,000/mm³ at first, but following the suspension of Prednisone the platelets persisted at levels below 100,000/mm³ Tw months after Imuran therapy she developed leucopenia but the leucocytes were restored to normal after the reduction of Imuran to 50 mg daily (2.5 mg/kg/day) On July 18, 1967 i.e. after 9 months, Imuran was completely

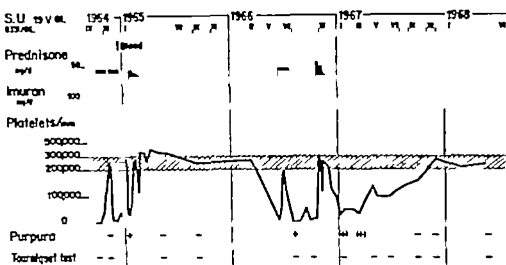


Fig. 5. Case 4. Platelet response following use of Prednisone and Imuran therapy. Platelet counts gradually increase to above 150,000/mm³ for nearly one year after Imuran was suspended.

discontinued and the platelets, without treatment, progressively rose to 270,000/mm³ in November 1967. They have remained above 200,000/mm³ since (fig. 5).

Comment

Acute idiopathic thrombocytopenic purpura (ITP) frequently runs a self-limited course. The incidence of lasting remissions, with or without treatment, varies from 50 to 80% [8, 9, 14, 30, 37, 45, 46, 50] and between 10 to 20% become chronic [30, 37, 50]. Corticosteroids and splenectomy have so far been recognised to be the treatment of choice in chronic ITP. However, evidence is lacking that corticosteroids can produce a complete remission, even when given for years, and there is no doubt that in most cases, splenectomy cannot ultimately be avoided. Plasma infusions are worthy of trial before splenectomy [43, 44] and immunosuppressive agents have been claimed, especially among adults, to have therapeutic effects in chronic refractory ITP.

The chronic cases may commence in the same manner as the self-limited acute type. In a series of 55 patients, WINTROBE [52] found that the self-limited cases were all under 10 years of age, and from the histories of these children, one could not have predicted in advance towards which direction their course might run. Remissions and

relapses belong to the natural history of the chronic form. If one overlooks the possibility of spontaneous remissions and makes observations on small numbers of cases for a short period of time, it becomes possible to make a case for many therapeutic agents [WINTROBE, 1967] Therefore, we kept our 4 patients with chronic or frequently relapsing ITP under close observation for 9 months to 2 years in order to be certain that no spontaneous remission occurred before Imuran therapy was started.

Corticosteroid therapy This is well established in the management of acute ITP Among our 61 patients treated with steroids from 1952 to 1965 22 of them (36%) have achieved clinical remission. In a series comprising patients of all ages, corticosteroid therapy was successful in approximately 30% [9 52] On the other hand, up to 73% remission rates associated with corticosteroid therapy have been reported [12, 13] This variation is most probably due to differences in selection of cases.

In chronic cases, corticosteroids may be effective in large doses but their long term administration results in undesirable effects as in case 1 and 4 The major hazard in children is retardation of growth as demonstrated in case 1 (fig 2) prior to the steroid therapy the velocity of his growth was on the 50th percentile whereas throughout the course of treatment his growth velocity dropped to below the 3rd percentile. Nevertheless, it returned to the pre-treatment level and for a short period rose even higher – catch-up growth [38] – when the prednisone was discontinued. In contrast, Imuran had no effect on his growth. It has been shown that corticotrophin does not interfere with growth to the same extent as exogenous steroids while at the same time controlling the underlying disease [17 18] In rare cases of ITP that were refractory to Prednisone, some observers have recorded success with ACTH [6] But, since it has to be given parenterally thus involving the risk of local haemorrhage, we feel that ACTH should be avoided. In keeping with other well known adverse effects of steroids, it has been noted that their long-continued administration may cause suppression of platelet formation, thus preventing the occurrence of a remission [11 42] Hence, the role of corticosteroids in the management of chronic ITP in children is limited although they may be indispensable in patients with acute severe exacerbations.

Imuran therapy Our experience in the treatment of chronic ITP with Imuran does not appear to be satisfactory since the initial rise in platelet counts was attributed to the large doses of Prednisone. In

case 1 and 2, no therapeutic effect whatsoever has been observed. In case 3 the patient was treated for approximately 10 months with Imuran. For the first 8 months the child seemed to respond to the treatment but during the last 2 months he relapsed. Should Imuran be discontinued after 6 months, one would have the impression that the immunosuppressive therapy was successful in this case. In case 4 it is doubtful that the patient was definitely improved on Imuran On the contrary the platelet counts steadily rose to over 200 000/mm after cessation of the immunosuppressive agent. Transient sharp rise of platelet counts was also observed in case 1 on two occasions after temporary discontinuation of Imuran and the thrombocytopenic purpura recurred when the treatment was resumed. RUNDLES [39] has observed one adult patient with thrombocytopenic purpura refractory to Prednisone and subsequently treated with B.W.57 323 (purine antagonist) He found that the drug initially appeared to depress the platelets even further but after cessation of the therapy there was a striking return of platelet count to normal levels within a fortnight which, however relapsed 2 weeks later Eventually a regimen of 3 days therapy every fortnight was worked out and thus succeeded in maintaining a remission. In our cases, however it is doubtful that intermittent Imuran therapy would have brought the platelet counts to normal levels. In case 1 the platelets remained low after 2 months cessation of therapy whereas in case 4 the platelet counts are maintained at levels above 150 000/mm³ for nearly one year after the immunosuppressive agent was suspended in July 1967

Several authors have reported more favourable results in the treatment of chronic refractory thrombocytopenic purpura, especially among adults (table I) However some of the patients studied had had splenectomy varying from 2 months to over 5 years when Imuran therapy was begun and in others, Imuran was administered only for a short period. Therefore, the therapeutic effects of immunosuppressive agents are not very conclusive and the reported successful rates in selected cases could in fact be attributed to the Prednisone or even to spontaneous remission. In children the experiences are still limited. Until now it seems that the results are poor but further fully controlled studies with immunosuppressive agents are needed.

Common *side effects of immunosuppressive agents* include anorexia, nausea, infection and bone marrow depression. These are dose dependent and are usually reversible on reducing dosage or on stopping the drug Much more rarely liver damage has been reported

[32] The teratogenic effects of immunosuppressive agents are well recognised and hence should be considered carefully when they are used in the treatment of human females in childbearing age [21 49 55] Although there is no evidence that drug induced immunosuppression is associated with an increased occurrence of spontaneous malignant tumours in man [48] this possible danger should, however require special consideration. Among our patients, case 1 and 4 developed leucopenia but the white cell counts returned to normal when the drug was temporarily discontinued as in case 1 or the dosage was reduced to half as in case 4 None of our 4 children has had severe infection other than transient rhinopharyngitis when they were on Imuran. Case 1 and 2 both developed jaundice with biochemical evidence of liver damage 3 to 4 months after being on Imuran therapy Their liver function tests, however normalised after the treatment was suspended. No liver biopsy was carried out in either case because of the presence of thrombocytopenia. Pruritus was rather troublesome in case 1 and 2 when they were on Imuran. It is unlikely that pruritus is due to the underlying disease as the symptom completely subsided when the immunosuppressive agent was discontinued. Nevertheless, it is a matter of conjecture whether pruritus is another side effect of immunosuppressive agents.

The effect of purine antagonists on immunoglobulins has been reported [33 48, 53 54] In 3 of our cases studied (table II) only case 1 showed a diminution of IgG and a slight diminution of IgM during treatment the other 2 cases showed insignificant reductions in IgG and/or in IgM. Hence no definite conclusion can be drawn whether reduction in immunoglobulins is a constant feature in patients receiving Imuran.

Furthermore, it is interesting to observe that the platelet adhesiveness was somehow influenced by the Imuran therapy and became normal when the treatment was interrupted (table III) According to SALZMAN [40] the values of platelet adhesiveness in adults occupy a normal distribution with a range of 26 to 60% whereas in children, according to our own observations, the normal distribution lies between 21 to 80 %. There is no correlation between platelet counts and platelet adhesiveness, but several factors are involved for platelets to become adhesive [25 35] Whether Imuran has a certain effect on the platelet surface on their metabolism, or membrane permeability and whether the influence of Imuran on the platelet adhesiveness has any clinical significance, will require further critical study

Table II. Immunoglobulin-assays in 3 cases of chronic idiopathic thrombocytopenic purpura treated with Imuran

	Case 1	Case 3	Case 4
IgG, g%			
Before treatment	0.860	–	–
3 months after treatment	0.480	0.780	
6 months after treatment	0.780	1 160	0.950
9 months after treatment	–	1 170	–
9–12 months after discontinuation of treatment	1.000	1 180	1.000
IgA, g%			
Before treatment	0.240	–	–
3 months after treatment	0.100		
6 months after treatment	0.080	0.060	0.080
9 months after treatment	–	0.080	–
9–12 months after discontinuation of treatment	0.123	0.073	0.133
IgM g%			
Before treatment	0.100	–	–
3 months after treatment	0.050	–	–
6 months after treatment	0.030	0.032	0.200
9 months after treatment	–	0.064	–
9–12 months after discontinuation of treatment	0.078	0.092	0.123

Table III. Effects of Imuran-therapy on clot retraction and platelet adhesiveness

	3–9 months after treatment			3–6 months after discontinuation of treatment		
	Platelets per mm³	Clot retraction %	Platelet adhesiveness %	Platelet per mm³	Clot retraction %	Platelet adhesiveness %
Case 1	37,000	12.0	16.2	620,000	85	40.8
Case 2	37,000	0	24.0	220,000	80	48.0
Case 3	83,000	12.5	16.9	338,000	60	41.7
Case 4	88,000	33.0	27.3	132,000	85	41.8
Own normal values	200,000 – 300,000	100.0	21 28			

after splenectomy

Plasma infusions have been claimed to have therapeutic effect on thrombocytopenia [5 43 44] but in case 1 and 2 they failed to achieve a clinical or haematological remission.

Splenectomy was first advocated half a century ago by KAZNELSON. The marked thrombocytosis following splenectomy has been interpreted as an indication that the spleen inhibits the production of platelets from the megakaryocytes and their delivery into the circulating blood [45, 46]. In 3 of our 4 cases (1, 2 and 3) splenectomy has ultimately achieved a clinical as well as haematological remission for nearly one year. It is known that approximately 85% of the patients with chronic thrombocytopenic purpura recover after splenectomy [9, 52] and our experiences from 1952 to 1965 in this respect have shown the same remission rates [50].

Conclusions and recommendations for future therapy. The need of an effective, safe, and well-planned therapy for the treatment of chronic idiopathic thrombocytopenic purpura in children is evident. From our experience and based on the present available literature, we recommend the following management:

1. Prednisone 60 mg/m²/day for a maximum of 3 months when clinically indicated, otherwise no therapy but close observation.
2. Prednisone 120 mg/m²/day for 4 weeks maximum when no remission is achieved on Prednisone 60 mg/m²/day.
3. Transfusions of fresh plasma when 1. and 2. fail to produce a remission.
4. Splenectomy when the above measures do not lead to a remission after 6 to 12 months.
5. Repeat 1, 2, 3, or Imuran therapy (3–5 mg/kg/day) in cases who fail to respond to, or relapse after initially successful, splenectomy if further treatment is clinically indicated. The possibilities of complications in patients receiving Imuran have to be taken into careful consideration.

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Summary

Our experience in the management of chronic idiopathic thrombocytopenic purpura (ITP) in 4 children have been described. All of them responded to large doses of Prednisone but the therapeutic effectiveness of Imuran therapy was not observed. Adverse effects of long-term administration of Prednisone as well as Imuran therapy are briefly reviewed. Splenec

tomy was ultimately performed with satisfactory result in 5 patients, whereas the remaining one recovered spontaneously after cessation of Imuran therapy. On the basis of this study a plan for management of chronic ITP in children is proposed.

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On the Heterogeneity of Bence Jones Protein

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In 1964 we reported that when Bence Jones protein was added to urines which contained albumin the two proteins could be separated by filtering the mixture through columns of Sephadex 75 [10] In those experiments the amounts of Bence Jones protein added were approximately equal to the albumin content. We have more recently used the same filtration technique to separate the proteins present in the native urines of myeloma patients with marked Bence Jones proteinuria and faint or moderate albuminuria. For the analysis of the two proteins vertical acrylamide gel (Cyanogum 41) electrophoresis was used [7]

We have also estimated the degree of heterogeneity of Bence Jones protein by analyzing – both with paper and with acrylamide gel electrophoresis – the protein fractions of successive Sephadex filtrates.

The search for signs of heterogeneity ultimately led us to a review of the vagaries of precipitation of this polypeptide at varying pH's and temperatures.

Methods

1 *Vertical acrylamide gel electrophoresis* was performed in the apparatus described by Raymond. Apart from the use of tris-glycin buffer Raymond technique was followed in detail [7 8].

2. *Sephadex filtration.* The technique described in previous article [10] was followed again. However instead of pH buffer sodium phosphate buffer of pH was used for the preparation and elution of the Sephadex columns.

Results[1]

1 Differential Filtration of Albumin and Bence Jones Protein

In the course of Sephadex filtration of 20 urines which contained large amounts of Bence Jones proteins together with small amounts of

[1] For technical reasons not all curves can be reproduced. However 6 representative samples are included.

albumin, the electrophoretic bands of albumin, the larger molecule appeared in the early Sephadex filtrates whereas the bands of Bence Jones proteins, the smaller molecules, were visualized in later filtrates (fig 1 4 and 5) This difference was demonstrated better by acryl amide gel than by paper electrophoresis.

2. Heterogeneity of Bence Jones Proteins

a) Electrophoretic mobility of 10 Bence Jones specimens On paper electrophoretic strips 5 specimens travelled with the speed of γ globulin (fig 2, 4 and 5) Five specimens were faster and were found in the β area (fig 1 3 and 6)[*] On the acrylamide gel strips all γ Bence Jones proteins were slower than the ferritin bands The β Bence Jones proteins were found in the ferritin area or travelled even faster than ferritin

Five of our 10 specimens were immunologically κ chains (fig 1 2, and 6) three were λ chains (fig 3 and 5) The immunologic character of the two other specimens was not known. Three of the 5 κ (fig 2 and 4) and one of the 3 λ chains (fig 5) travelled with γ speed. The other 2 κ (fig 1 and 6) and 2 λ (fig 3) were found in the β areas. The antigenic group of the Bence Jones chains is decidedly not a factor which influences the speed of the specimens during either paper or acrylamide gel electrophoresis.

b) Acrylamide gel electrophoresis of fractions of Bence Jones proteins obtained by Sephadex filtration. before Sephadex filtration. In 3 specimens the Bence Jones proteins presented both in paper and acrylamide strips *mainly* as one-banded units. In contrast to the findings in the paper strips, in the acrylamide strips of 7 specimens (fig 1 to 6) multiple bands were found.

After Sephadex filtration. As explained in the legends of fig 1 to 6, several acrylamide patterns made before and during Sephadex filtra tion reveal clear-cut signs of heterogeneity Other specimens, however appear to be practically homogeneous. A few examples must suffice.

Bence Jones protein with molecular weight of pproximately 20,000 is identical or closely related to the light chain of γ globulin. Since globulin has molecular weight of 150,000 Bence Jones protein cannot be globulin. In this article the terms β and γ Bence Jones protein will be used to designat Bence Jones proteins which move during electrophoresis with the speed of either β or γ globulin.

The ferritin band, which is the most important point of the orientation on the acrylamide gel electrophoretic patterns of the serum, is situated in the middle of the acrylamide strip halfway between the γ globulin and albumin bands.

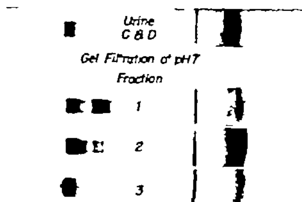


Fig. 1 Patient Pi, exp. 111 κ group, β speed. In the pre-filtration acrylamide strip bands are present. During filtration a faster third band ppears in the second eluate. This fast band should therefore have a smaller molecular weight than the 2 Bence Jones bands observed in the pre-filtration strip. This faster component is the only remaining band in the paper strip of the third eluat which also points in this direction.

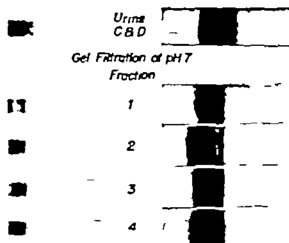


Fig. Patient Ka, exp. 97 κ group, γ speed. I the pre-filtration strip there are 3 bands closely together. In the fourth eluat the second band is far the heaviest. The slower band has disappeared and the faster band is much thinner. The middle band must therefore represent a smaller peptide molecule.

In figure 5 apart from 3 well-defined bands a faint faster and hardly visible slower band were present before filtration. In the fourth eluate

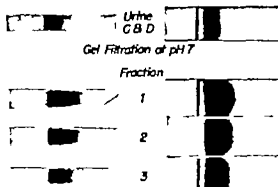


Fig. 3. Patient Ra, exp. 112: λ group, slow β speed. The 3 bands, which before Sephadex filtration differ markedly in intensity appear in the first tw eluates as 3 equally strong bands. Both in the first and in the second luates new strong faster band ppears, becomes much weaker in the third luate The latter band therefore has heavier molecular weight than the 3 original bands.

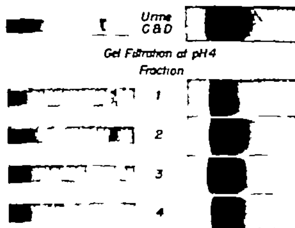


Fig. 4. Patient Spi, exp. 96 group, γ speed. The 4 adjacent, nearly confluent bands present in the prefiltration acrylamide strip are practically unchanged in all 4 eluates.

the 3 strong bands visible in the prefiltration strip are weaker but the weak slowest and fastest bands are stronger than before filtration. These two weak bands possibly have a smaller molecular weight than the 3 strong components.

In figure 6, 3 bands were present before filtration the slowest being the strongest. The new slow bands which are seen in the first two

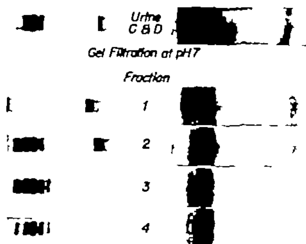


Fig 5. Patient Be exp. 105 λ group, γ speed. Three learest bands are present before filtration. I addition there is faint adjacent faster band and hardly visible adjacent slower band. The molecular weight of these 5 bands cannot be the same because in the third hiate all 4 slower bands are strong. The slowest band, which was hardly visible in the pre-filtration strip, is stronger than the fifth and fastest band. In the fourth ehiat where the 3 strong components of the pre-filtration strip have become weaker both the slowest and the fastest band are still more ma ked than in the pre-filtration strip.

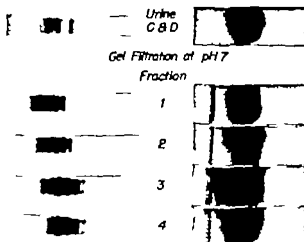


Fig 6. Patient Pe exp. 100 κ group, β speed. Before filtration 3 bands are present in the acrylamide strip, the slowest being the strongest I the first 2 filtrates new slow bands ppear re absent in the fourth hiat These new components must have larger molecular weight than the original bands. I addition in the third and fourth filtrates 2 new strong fast bands ppear They must be lighter than the bands seen in the original strip.

filtrates but are not present anymore in the fourth filtrate may represent components with a larger molecular weight. Two new strong fast bands which appear in the third and fourth filtrates must be lighter than the bands seen in the pre filtration strip

In other specimens fewer manifestations of heterogeneity were found. For instance in figure 4 the 4 adjacent, nearly confluent bands present in the acrylamide strip made before Sephadex filtration can be distinguished almost unchanged in all 4 eluates.

c) Precipitation and solution of Bence Jones protein at different temperatures Some recently published studies have mentioned that Bence Jones proteins with the same sedimentation constant i.e. the same molecular weight and identical antigenicity might "slightly" differ in precipitation and solution at higher temperatures [2] These variations are more intense than previously assumed.

1 All Bence Jones proteins precipitate at a pH of 4.9 if heated to a temperature of 56–58° C [5] At this pH, however certain specimens precipitate at a temperature as low as 45 C, whereas others have to be heated for at least 5 min at 56° C.

2 Part of the Bence Jones proteins will demonstrate, even at pH 4.9 the so-called Bence Jones phenomenon the protein goes into solution at boiling and precipitates at cooling At this pH other specimens, however do not dissolve when boiled and some of those which do dissolve at 100° C do not precipitate on cooling

3 Although it is widely believed that at a pH between 1 and 2 Bence Jones proteins always dissolve at boiling and always precipitate at subsequent cooling, nothing could be less true. Bence Jones proteins, if mixed with HCl or HNO solutions of pH 1 to 2, do not show the Bence Jones phenomenon anymore.

Aliquots of 4 ml of Bence Jones protein solutions were precipitated at a pH of 4.9 and 56 C. After centrifugation the supernatants were discarded and the 4 precipitates (a, b, c, and d) were suspended in (a) 4 ml of pH 2 HCl buffer (b) 4 ml pH 1.3 HCl buffer (c) 4 ml pH 1 0 HCl buffer (d) 4 ml of HNO 0.5 (pH 1 0) In each of these tubes the Bence Jones protein precipitate went into solution. This happened in certain specimens already at room temperature, in others heating to 30° C to 60° C and in a few cases boiling was necessary to dissolve the precipitated Bence Jones protein.

In none of the Bence Jones proteins which had cleared after boiling did a precipitate form when the solution was cooled. To these 4 clear solutions of Bence Jones protein dissolved in HCl or HNO of pH 1

which were negative as far as the Bence Jones phenomenon was concerned, equal amounts of 3 sulfosalicylic acid [4] were added. The pH of the solutions hardly changed and varied between 1 and 1.2. Thereafter the Bence Jones phenomenon could be elicited again. (1) The sulfosalicylic acid caused a precipitate in all tubes. (2) At boiling the precipitate went into solution to reprecipitate at cooling. These tests were performed with 20 different Bence Jones proteins.

Sulfosalicylic acid evidently changes the Bence Jones protein in such a way that the manifestations of heterogeneity disappear. Thereafter the differences in behavior of different Bence Jones proteins at boiling and cooling no longer occur.

Discussion

Differences in serologic antigenicity and in velocity during electrophoretic analysis favor the concept that Bence Jones proteins are heterogeneous. Furthermore methionine is present in the κ and absent in the chains and both amino acid sequences and end groups of Bence Jones protein show considerable variations from case to case [6]. Variations have also been observed in the optical parameter of Bence Jones protein [13]. Whereas the light chains of normal γ globulins present in starch electrophoresis as one or maximally two bands, Bence Jones proteins very often appear as 4 or 5 bands. In this connection the polymerization of Bence Jones protein must also be mentioned [3].

We found that electrophoretic analysis of the successive eluates obtained during filtration of Bence Jones proteins through Sephadex columns provided additional evidence of heterogeneity. The possibility that the combination of concentration, Sephadex filtration and acrylamide could have changed certain characteristics of Bence Jones protein cannot be completely disregarded. It could also be argued that the multiple bands present in the gel electrophoretic curve of the native urine represented proteins other than Bence Jones protein, especially globulins. However after Sephadex filtration, the final fractions can contain only molecules of the size of Bence Jones protein because the larger molecules have been removed in the first filtrates. Actually starch gel patterns of Bence Jones protein published by others [11] are similar to the acrylamide patterns found in our curves.

The differences of behavior of Bence Jones proteins if suspended

in HCl and HNO_3 solutions of pH 1 to 2 are another sign of heterogeneity. These differences disappear after addition of sulfosalicylic acid. Hereby the Bence Jones phenomenon which had disappeared under influence of HCl and HNO solutions with a pH of 1 to 2 became positive again. In view of the disappearance of heterogeneity of Bence Jones protein after addition of sulfosalicylic acid the use of this acid for the determination of Bence Jones protein should be warmly recommended in order to obtain greater accuracy [9].

VAN EYCK and MONFOORT [11] have correctly emphasized that Bence Jones protein eliminated in multiple myeloma is the light chain of *pathological* and *heterogeneous* myeloma γ globulins. Therefore the light chains of these myeloma globulins, i.e. the Bence Jones protein excreted in the urine could hardly be expected to be homogeneous.

Notwithstanding our emphasis on the importance of the Bence Jones phenomenon, the presence of this polypeptide is certain only if apart from dissolving at boiling and reprecipitating at cooling the protein under investigation also precipitates at a temperature of 56–56 C at a pH of 4.9. Ultimately confirmation by electrophoresis is necessary.

Summary

There is no parallelism between velocity in the electrophoretic patterns and antigenicity Bence Jones protein. The large molecules present in the banded gel electrophoretic patterns of many Bence Jones protein specimens are eliminated in the earlier Sephadex eluates. In the terminal eluates where only small molecules like Bence Jones protein can be present, the banded character of the polypeptide is often more marked than in the native urine. The changing of the bands is an indication of heterogeneity. Bence Jones proteins brought pH varying between 1 and 2 by HCl and HNO_3 solutions, behave in many different ways but the Bence Jones phenomenon is always negative. Bence Jones proteinuria should only be diagnosed if, (1) after addition of sulfosalicylic acid the Bence Jones phenomenon is present, (2) the protein precipitates at 56° at pH of 4.9

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Untersuchungen an Nukleohistonen

VI. Vergleichende Untersuchungen des Chromatin aus leukämischen Zellen und aus normalen Granulozyten

P. Drings und E. Harbers

Als Begleiterscheinung der Cancerogenese kommt es meist zu sogenannten Deletionen, d.h. in den Tumorzellen fehlen verschiedene Enzyme (oder sie sind nicht mehr induzierbar), welche in den normalen Ursprungszellen vorhanden sind. Im Laufe der Zeit wurden verschiedenartige Deutungen für die Ursache solcher Deletionen gegeben, u.a. wurden sie auf somatische Mutationen zurückgeführt. Eine neue Erklärungsmöglichkeit gründet sich darauf, dass in einer Reihe von Tumoren eine erhöhte Heterochromatisierung beobachtet wurde [9]. In somatischen Zellen ist nur ein Teil der genetischen Information im Zellkern, das sogenannte Euchromatin, funktionell verfügbar, die übrigen Informationsanteile im dichtgepackten Heterochromatin sind – wahrscheinlich weitgehend permanent – ausgeschaltet. Eine Zunahme des Heterochromatins auf Kosten des Euchromatins, wie sie in Tumorzellen nachgewiesen wurde, muss zu funktionellen Ausfällen führen. Auch in leukämischen Zellen wurden Deletionen beobachtet, von denen einige zur Möglichkeit einer bevorzugten Schädigung dieser Zellen durch bestimmte Antimetabolite wesentlich mit beitragen. Wenn als Deletion ein Verlust des Enzyms Asparagin-Synthetase vorliegt, kann sogar eine spezifische Therapie durch Verabfolgung einer aus *E. coli*-Zellen isolierten Asparaginase durchgeführt werden [5, 11].

Mit den nachstehend geschilderten Untersuchungen sollte geprüft werden, ob auch in leukämischen Zellen (akute Leukämien und chronische myeloische Leukämien) eine gesteigerte Heterochromatisierung nachzuweisen ist, wie sie zuvor in verschiedenen Tumoren [7, 10, 13] festgestellt worden war. Als Vergleichsbasis dienten die Analysewerte von normalen Granulozyten und Lymphozyten.

Material und Methodik

Es wurde das Blut von je 5 Patienten mit akuten Leukämien und mit chronischer myeloischer Leukämie untersucht die wichtigsten klinischen Daten sind in den Tabellen I und II zusammengefaßt. Durch spontane Sedimentation (60 min in Schrägstellung von 45° bei einer Temperatur von 37°C und 30 min in Senkrechtstellung bei Zimmertemperatur) wurden aus 20 bis 80 ml Blut – gemischt mit 6%igem Dextran (Macrodex®) im Verhältnis 4 1 und 25 Einheiten Heparin pro ml (Liquemin®) – die Leukozyten angereichert und aus dem erythrozytenreichen Überstand vorsichtig abpipettiert. Von der so gewonnenen Leukozytensuspension wurde ein kleiner Teil für Zellzählung und DNS-Analyse entnommen, um den mittleren DNS-Gehalt pro Zelle zu bestimmen. Die übrigen Leukozyten wurden 30 min bei 37°C inkubiert dann mit gepufferter isotonischer N Cl-Lösung gewaschen und mit Hilfe des Homogenisators nach EMANUEL und CHARGAFF [5] homogenisiert. Durch Zentrifugieren bei 600 *g* ließen sich die Zellkerne sedimentieren. Die Fraktionierung des Zellkerninhalts in eu- und heterochromatinreiche Fraktionen sowie eine Misch-Fraktion erfolgte nach dem Prinzip des von FRENSTER *et al.* [4] angegebenen Verfahrens durch differenziertes Zentrifugieren Einzelheiten der Methodik wurden an anderer Stelle beschrieben [2 8]. Für die quantitative DNS-Analyse wurden die Nukleinsäuren aus den Chromatinfraktionen extrahiert [14] und colorimetrisch mit Hilfe der Diphenylaminreaktion [1] bestimmt. Bei der Auswertung wurde die DNS der Mischfraktion (~10% der Gesamt-DNS) jeweils zur Hälfte den beiden Chromatinarten zugerechnet. Die so gewonnenen Analysewerte zur Verteilung der DNS auf Eu- und Heterochromatin sind Relativwerte; die Streuungsbreite beträgt ± 5 bis gelegentlich ± 10%. Eine absolute Mengenbestimmung der DNS in den beiden Chromatinarten ist zur Zeit noch nicht möglich [vgl. 8]

Die von den leukämischen Zellen erhaltenen Ergebnisse sind denen entsprechender Untersuchungen an normalen Granulozyten und Lymphozyten gegenübergestellt. Bei dem Blut von 7 gesunden Spendern und 9 Patienten, deren Differentialblutbild eine Linksverschiebung zeigte wurden die Leukozyten in der oben beschriebenen Weise gewonnen, dann jedoch mit Hilfe des von GARVIN [6] angegebenen Verfahrens in Granulozyten und Lymphozyten aufgetrennt. Eine genauere Beschreibung dieser Methode findet sich in einer vorangehenden Arbeit [2]. Die weitere Aufarbeitung (Kernisolierung und Fraktionierung des Kerninhaltes) erfolgt anschließend in der gleichen Weise wie bei den leukämischen Zellen.

Ergebnisse

In den Granulozyten aus normalem Spenderblut fand sich 53% der DNS im Eu und 47 % im Heterochromatin bei jüngeren Zellpopulationen (Linksverschiebung im Differentialblutbild) war die DNS-Menge im Euchromatin erhöht (bis zu 66 %) unter entsprechender Minderung der DNS im Heterochromatin Die Werte für normale Lymphozyten sind 32 % DNS im Eu und 68 im Heterochromatin [2] Verwendet man diese an normalen Leukozyten gewonnenen Werte als Vergleichsbasis, so ergibt sich für die untersuchten leukämischen Zellen meist eine beträchtliche Erhöhung der euchromatischen DNS-Anteile. Eine solche war bei allen 5 chronischen myeloischen Leukämien zu beobachten (Abb. 1) Ausnahmen waren 3 Fälle der

Tabelle I. Akute Leukämien

Patient	Alter Jahre	Geschlecht	Diagnose	bek. Dauer der Krankheit	Therapie	Leukozyten pro mm³	Differentialblutbild
Gö.	41	w	akut Retikulose	2 Wochen	keine	17 200	64% Blasten, 4% Promyel. 1% Myel., 1% Stabk., 6% Segm., 1% Eos. 1% Baso. 15% Lympho., 7% Mono.
Pr	31	w	leukäm. verl. generalisierte Lympho-Retikulose	2 ½ J	Amethopterin 6-Mercaptopurin Cyclophosphamid Vincristin	74 400	90% Blasten, 4% Stabk., 5% Segm., 1% Eos.
Schn.	$^{5}/_{12}$	m	Paramyeloblasten-leukämie	1 Woche	keine	48 200	35% Paramyel., 6% Stabk. 41% Segm., 18% Lympho.
Wi.	37	m	akute Leukämie Bronchial-Ca.	8 Wochen	Tele-Kobalt-Bestrahlung der Lunge R6, Halslymphkn. Proresid 6-Mercaptopurin	33 000	93% Blasten 5% Stabk., 2% Segm.
Ku.	67	w	subakute myeloische Leukämie	1 Woche	keine	178 000	37% Blasten 12% Promyel., 7% Myel 8% Metamyel., 15% Stabk., 21% Segm.

Tabelle II Chronische myeloische Leukämien

Patient	Alter Jahre	Geschlecht	bek. Dauer der Krankheit	Therapie	Leukozyten mm³	Differentialblutbild
He	65	w	13 Jahre	Busulfan, ³²P	38 900	4% Myel., 6% Promyel., ■ Myel., 19% Met.myel., 9% Stabk., 40% Segm., 2% Bas., 2% Eos., 4% Lymph.
Ra	39	m	8 Monate	Busulfan	24 700	6% Myel., 29% Promyel., 1% P rald st, 11% Myel., 1% Met.myel., 5% Stabk., 43% Segm., 1% Bas., 3% Lymphoz.
Da.	.9	w	4 Wochen	keine	100 000	1% Blasten, 8% Promyel., 5% Myel., 14% Metamyel., 36 Stabk., 32% Segm., 2% Lymphoz., 2% Mono.
Bu.	72	w	2½ Jahre	Busulfan	8 500	23% Blasten (% Promy.), 9% Myel., 2% Metamyel., 14% Stabk., 13% Segm., 23% Bas., 2% Eos., 4% Lymphoz., 1 Mono., 3% nicht einzuordnen
Her	34	m	9 Jahre	Busulfan, Rö. Milz, Methylhydrazin, 6-Mercaptopurin	10 500	8% Blasten, 47 Promyel., 11% Myel., 2% Metamyel., 7% eos. Myel., 7% Stabk., 10% Segm., 2% Bas., 5% Lymph., 1% nicht einzuordnen

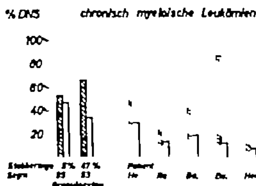


Abb. 1 Prozentualer Anteil der DNS in der Euchromatin- (schraffierte Säulen) und in der Heterochromatin-Fraktion (offene Säulen) der weissen Blutzellen von 5 Patienten mit chronischer myeloischer Leukämie. Zum Vergleich sind links die entsprechenden Analysewerte von normalen Granulozyten wiedergegeben. Die Prozentwert für stab- und segmentkernige Zellen gelten dabei für die isolierten Granulozyten.

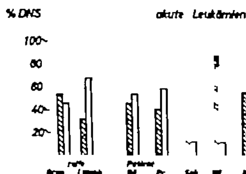


Abb. 2 Prozentualer Anteil der DNS in der Euchromatin- (schraffierte Säulen) und in der Heterochromatin-Fraktion (offene Säulen) der weissen Blutzellen von 5 Patienten mit akuter Leukämie. Zum Vergleich sind links die entsprechenden Analysewerte von isolierten Granulozyten und Lymphozyten wiedergegeben.

akuten Leukämien (Abb 2) bei diesen handelte es sich um 2 Retikulosen (Pat. Gö. und Pr) und um eine subakute chronische Myelose (Pat. Ku.) Der durchschnittliche DNS-Gehalt pro Zelle wurde für normale Leukozyten mit $5{,}4 \times 10^{-6}$ µg ermittelt die leukämischen Zellen zeigten keine signifikanten Abweichungen, so dass die Möglichkeit von – zumindest grösseren – Veränderungen im Ploidiegrad ausgeschlossen werden konnte.

Die mit der Auftrennung der normalen weissen Zellen in Granulozyten und Lymphozyten verbundene Aufarbeitung beeinflusst etwas die spätere Fraktionierung des Inhalts der Zellkerne [2] durch die Adsorption an silikonisierte Glasperlen kommt es offenbar doch zu einer gewissen Schädigung der Zellen welche eine geringe Vermehrung der in der Heterochromatinfraktion erscheinenden DNS zur Folge hat. Die Berücksichtigung dieses Fehlers ändert jedoch nichts an den in Abbildung 1 und 2 wiedergegebenen Ergebnissen Eine Auftrennung der leukämischen Leukozyten an silikonisierten Glasperlen ist *nicht möglich* [12] und so musste auf eine Fraktionierung dieser Zellen (die allerdings meist in recht hoher Konzentration vorlagen vgl Tabelle I und II) verzichtet werden

Diskussion

Für Untersuchungen der Art, wie sie hier vorgenommen wurden, ist eigentlich Voraussetzung dass pathologisch veränderte und normale Zellen aus denen erstere entstanden sind, vergleichend analysiert werden. So sollten Befunde von leukämischen Zellen korrekterweise mit denen von ihnen entsprechenden Leukozytenvorstufen verglichen werden. Im Rahmen biochemischer Untersuchungen ist diese Forderung allerdings nicht realisierbar und so blieb nur die Möglichkeit eines Vergleiches der Analysewerte von leukämischen Zellen mit denen von normalen Leukozyten.

Zu einer Einschränkung in der Verfügbarkeit der genetischen Information durch eine Zunahme des dichten Chromatins bzw eine Minderung der Euchromatinanteile kommt es natürlicherweise mit fortschreitender Differenzierung eines Gewebes Die im Kreislauf zirkulierenden segmentkernigen Granulozyten sind der Endzustand solcher Entwicklung mit einer Linksverschiebung vermehrt erscheinende stabkernige (jüngere) Zellen zeigen eine vergleichsweise höhere Euchromatisierung [2] welche *in den Vorstufenzellformen noch weit* ausgeprägter sein dürfte Die in den leukämischen Zellen gefundenen *hohen Werte für die Euchromatinanteile sind daher als Zeichen dafür* zu werten, dass die zur Leukämie führenden Veränderungen bereits auf einer frühen Stufe der Zellreife erfolgen Die Frage ob die in leukämischen Zellen beobachteten Deletionen über eine Ausschaltung genetischer Information durch Heterochromatisierung zustande kommen kann auf Grund der vorliegenden Untersuchungen noch nicht

beantwortet werden. Mit den Analysewerten von normalen Granulozyten als Vergleichsbasis wäre nur ein Hinweis für eine positive Antwort möglich gewesen, und zwar dann wenn die leukämischen Zellen in ihrer Bruttobilanz eine noch weiter gesteigerte Heterochromatisierung gezeigt hätten dagegen ist eine klare negative Antwort bei dem gewählten Versuchsansatz nicht zu erhalten. Die Klärung des Problems mag vielleicht über einen methodisch anderen Weg der zur Zeit versucht wird, möglich werden. Durch vergleichende Bestimmung des Ausmaßes der Hybridisierung von DNS aus Eu und Heterochromatin läßt sich die Information in den Kernfraktionen qualitativ charakterisieren. Allerdings gibt es auch hier methodische Einschränkungen Sofern die entscheidenden Informationsanteile nur sehr kleine Bereiche umfassen, ist das Auflösungsvermögen nicht hinreichend, um deren Heterochromatisierung noch zuverlässig nachweisen zu können.

Die Untersuchungen wurden durch eine Beihilfe der Deutschen Forschungsgemeinschaft ermöglicht, ferner durch Gewährung von Personalmitteln vom Bundesministerium für wissenschaftliche Forschung gefördert. Für die Überlassung der Blutproben von Leukämiepatienten danken wir den Herren Prof. W. Creutzfeldt (Medizinische Universitätsklinik Göttingen) Prof. G. Joppich (Universitäts-Kinderklinik Göttingen) Prof. F Heckner (Stadt- und Kreiskrankenhaus Einbeck) und Mitarbeitern. Frau U Spaar und Frl. B. Schrader danken wir für zuverlässige technische Hilfe.

Zusammenfassung

Aus den Leukocyten von 5 Patienten mit akuter Leukämie und 5 Patienten mit chronischer myeloischer Leukämie wurden unter standardisierten Bedingungen nach der von Frenster *et al.* entwickelten Methode eu- und heterochromatinreiche Kernfraktionen isoliert, ihr Gehalt an Desoxyribonukleinsäure (DNS) bestimmt und mit den entsprechenden Analysewerten der Granulocyten und Lymphocyten gesunder Blutspender verglichen. Bei unveränderter DNS-Menge pro Zellkern war in den leukämischen Zellen das Mengenverhältnis von Eu-/Heterochromatin im Vergleich zu den normalen Leukocyten im Sinne einer erhöhten Euchromatisierung verschoben.

Summary

The cell nuclei of leukocytes from 5 patients with acute leukemia and from 5 patients with chronic myeloid leukemia were fractionated according to the method developed by Frenster *et al.* The derived eu- and heterochromatin-rich fractions were analyzed for their DNA-content and the results compared with the corresponding values of normal granulocytes and lymphocytes. In the leucemic cells the DNA-content per nucleus was found to be unchanged while the ratio of the DNA in eu-/heterochromatin was increased as compared to normal leukocytes.

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Université de Liège

Ultrastructure des plaquettes dans deux cas de thrombocythémie

L. J. Simar et J. Hugues

La présence de modifications histologiques pathologiques dans les plaquettes peut difficilement être investiguée au moyen du microscope optique. Quoique le microscope électronique semble à priori un instrument plus approprié à ces recherches, il n'a permis cependant jusqu'à présent que la publication d'un nombre assez peu élevé d'études, dont on trouvera une synthèse dans le livre récent de Schulz [11]. Les travaux consacrés plus particulièrement aux thrombocythémies ne sont pas nombreux. Si Rebuck *et al.* [9] ont étudié des plaquettes de patients atteints de thrombocythémie primitive ou secondaire, ils n'ont pu cependant y déceler aucune anomalie ultrastructurale. Ce fait pourrait être dû à la technique d'étalement qu'ils ont utilisée. C'est essentiellement à Jean et à ses collaborateurs [1, 4, 6, 7] que nous devons les premières observations détaillées de l'ultrastructure des plaquettes dans certaines thrombocythémies. Dans leur excellent article, le plus récent [5], ils ont résumé leurs observations, portant sur 15 cas de thrombocythémie ou de thrombocytose parmi lesquels on note 4 cas primitifs certains. S'ils constatent la présence systématique d'anomalies ultrastructurales plaquettaires, ils ne peuvent cependant dégager de leurs études des critères permettant de différencier morphologiquement les atteintes primitives des secondaires. Notre travail n'a d'autre but que de verser au dossier de l'étude ultrastructurale des thrombocythémies deux nouvelles observations que nous avons réalisées et de comparer nos images à celles décrites par Jean *et al.*

Matériel et méthode

Nous nous sommes inspirés de la technique de Femly *et al.* [2]. Le sang périphérique est directement prélevé dans une seringue siliconée contenant déjà le fixateur et l'anticoagulant.

La proportion à respecter est de 1 partie de sang pour 1 partie d'EDTA à 1 et 8 parties de fixateur. Ce dernier est de la glutaraldéhyde diluée à 6 dans du tampon phosphate à pH 7,4. Le mélange est centrifugé pendant 10 min à 1600 g à température ordinaire. On recueille le surnageant qui subit une nouvelle centrifugation de 5300 g pendant 20 min toujours à température ordinaire. Le culot de plaquettes est recueilli, il est divisé en petits fragments ayant maximum 1 mm de coté; ceux-ci sont alors post-fixés pendant 1 h à 4 C dans de l'acide osmique dilué à 1% dans du tampon phosphate à pH 7,4. Après déshydratation, les blocs sont inclus à l'Epon. Les coupes sont colorées pendant 30 min dans une solution aqueuse d'acétate d'uranyle à 5 ‰ puis pendant 20 min dans du citrate de plomb à pH 1 selon Reynolds [10]. L'examen des spécimens se fait à l'Elmiskop I Siemens. L'étude comparative des plaquettes normales et celle des plaquettes pathologiques ont comporté l'examen sur photographies d'un minimum de 1000 thrombocytes par cas et une étude statistique des résultats a été pratiquée.

Etude clinique des cas

L'histoire clinique de ces deux patients, leur symptomatologie et les résultats des examens de laboratoire qu'ils ont subis ne font pas l'objet de notre travail. Nous nous contenterons donc de résumer dans le tableau I les principales données en rapport avec leur affection sanguine.

Résultats

Anomalies plaquettaires quantitatives

Les plaquettes des thrombocythémies contiennent des constituants qui peuvent ne présenter aucune anomalie ni de forme ni de structure mais qui pourraient être en proportion anormale. Ces faits n'apparaîtront qu'après numération et mensuration de ces principaux constituants. Nous avons calculé le nombre moyen par plaquette de granulations denses (granulomère α), de mitochondries (granulomère β) et d'inclusions lipidiques. La taille des vacuoles, la richesse en glycogène et la présence d'amas de vacuoles d'origine golgienne (granulomère γ) ont également retenu notre attention. Nos résultats sont schématisés dans le tableau II.

Il ressort de cette étude que le cas 2 a des chiffres comparables à ceux d'un sujet normal. La faible diminution du nombre des mitochondries n'est pas statistiquement significative. Dans le cas 1 il faut retenir les anomalies du système vacuolaire. En effet, d'une part on observe une assez grande fréquence de vacuoles géantes et d'autre part les amas de vacuoles d'origine golgienne sont nettement augmentés en nombre. L'augmentation du chiffre moyen de granulations denses s'est avérée statistiquement non significative.

Tableau I

	Cas 1	Cas 2
Age ans	64	34
Plaquettes/mm³	1 100 000	1 200 000
Leucocytes/mm³	13 300	11 000
Formule leucocytaire	Polynucléose neutrophile	Normale
Erythrocytes/mm³	4 350 000	3 000 000
Myélogramme	Réaction myéloïde Mégacaryocytes en nombre normal	Myélosclérose
Biopsie de crête iliaque	Hyperplasie des lignées myéloïde et mégacaryocytaire	Myélosclérose
Biopsie splénique	Hyperplasie réticulée Disparition des follicules lymphoïdes. Infiltration par des cellules indifférenciées	Hyperplasie des cellules réticulées
Symptomatologie	Thromboses depuis 4 ans. Hémorragies	Asthénie
Diagnostic	Thrombocythémie primitive	Thrombocythémie accompagnant une réaction myéloproliférative
Traitement	Myleran	Myleran
Evolution du nombre de plaquettes après traitement, par mm³	213 000	160 000

Anomalies plaquettaires qualitatives

Les modifications les plus importantes portent sur les granulations denses. Chez l'individu normal, celles-ci sont de forme généralement sphérique ou ovoïde et leur taille varie peu d'un élément à l'autre. Ce n'est qu'exceptionnellement qu'on observe de rares granulations allongées, en forme de bâtonnets.

Par contre, dans nos deux cas, nous avons rencontré un beaucoup plus grand polymorphisme de ces granulations (fig 1). La forme allongée, dite en bâtonnet, n'est pas rare (fig 1 et 2). Fréquemment, la granulation se prolonge par une sorte de manchon, ce qui lui donne un aspect de massue (fig 3 et 4). On observe également des granula

Tableau II

	Sujet normal	Cas 1	Cas 2
Nombre moyen de granulations denses par section de plaquett	4,04	6,90	4,82
Nombre moyen de mitochondries par section de plaquett	2,1	1,8	1,6
Nombre moyen d inclusions lipidiques par section de plaquett	0,2 %	0 1 %	0 %
Plaquettes ne contenant aucune vacuole	18%	27	17
Plaquettes contenant de petites vacuoles d'une taille d'environ 100 nm	80,5 %	63	80%
Plaquettes contenant des macrovacuoles de taille supérieure à 850 nm	1,5	10%	3%
Plaquettes ne contenant pas de glycogène visible	9%	12%	8
Plaquettes contenant du glycogène dispersé	74 %	79	81 %
Plaquettes contenant des amas de glycogène	17 %	9%	8
Amas de vacuoles d'origine golgienne	1	7	1

La formule

$$t = \frac{m1 - m_2}{m1 + m2}$$

nous donne pour le cas 1 t 0,86 non significatif au seuil de 5% ; pour le cas 2 : t = 0,26 non significatif au seuil de 5 %

tions en «cocarde» qui semblent constituées de plusieurs zones con centriques (fig 2)

Les modifications du système vacuolaire constituent la seconde anomalie qui apparaissait d ailleurs déjà dans le tableau II Chez l homme normal les vacuoles ont un diamètre qui dépasse rarement celui d une granulation dense c est-à-dire de 100 à 200 nm. Par contre dans nos deux cas de thrombocythémie mais surtout dans le cas 1 la taille des vacuoles augmente (fig 5) D assez nombreuses plaquettes contiennent de volumineuses vacuoles d un diamètre supérieur à 850 nm fig 6) Parfois même la plaquette n est plus constituée que de 2 ou 3 de ces énormes dilatations

Enfin, les amas de microvacuoles d origine golgienne (fig 7 ont été fréquemment observés dans le cas 1



Fig 1 Thrombocythémie secondaire. Important polymorphisme des granulations denses. Granules en bâtonnets (flèches) et en masse (flèche double) (× 12600)



3

Fig. — Thrombocythémie secondaire. Granulations denses en bâtonnets et en cocarde. [illegible]

Fig. 3. Thrombocythémie primitive. Granulations denses en masse. Nombreuses vésicules [illegible] [illegible]

4

5

Fig 4 Thrombocythémie primitive. Section de nombreuses granulations en masse (flèches) (× 52 000).

Fig 5 Thrombocythémie secondaire. V vacuolisation ( ) de l'hyalomère m = microtubules (34 000



Fig. 6. Thrombocythémie primitive. Dilatation vacuolaire plaquettaire (× 32000).

Fig. 7. Thrombocythémie primitive. Amas de vésicules d'origine golgienne (× 56000).

Discussion

Dans nos deux cas, la modification la plus importante concerne les granulations denses dont le polymorphisme est important L existence de granules allongés est connue dans diverses thrombopathies [8] Il en est de même pour les granulations en massue [8] Ces dernières sont particulièrement nombreuses dans la maladie de von Willebrand-Jürgens ou elles ont d ailleurs été découvertes pour la première fois [12] Elles ne sont donc pas spécifiques des thrombocythémies dans lesquelles elles ont été retrouvées par Jean et al [6] On ne connaît pas jusqu à présent la signification exacte de ces anomalies Quant aux granulations en «cocarde» que nous observons dans nos deux cas, elles ont été retrouvées tant dans les thrombocythémies primitives que secondaires et considérées comme un reflet d un défaut de thrombopoïèse [5] Enfin le nombre moyen de granulations par plaquette est augmenté dans notre étude sans toutefois que cette élévation soit statistiquement significative Les seules données numériques de la littérature sur les modifications de ce nombre plaident plutôt, à l'inverse des nôtres, pour une diminution dans les thrombocythémies [5]

L apparition de vacuoles volumineuses, second facteur qui caractérise nos deux cas, n a pas été signalée jusqu à présent. Lechner et al [8] ont mentionné une vacuolisation des plaquettes chez un thrombasthénique mais il s agissait d une augmentation du nombre et non de la taille des vacuoles. Quant à l accumulation de vésicules d origine golgienne, elle serait un indice d une perturbation de la thrombopoïèse [5] Nous ne l avons cependant retrouvée que dans un cas. Elle n est donc pas un facteur constant.

De nos deux cas, le premier était cliniquement une thrombocythémie primitive, le second une atteinte secondaire. Pas plus que les autres auteurs, nous n avons pu dégager de différences ultrastructurales notables entre ces deux cas, permettant d envisager un diagnostic différentiel.

Résumé

L'ultrastructure des plaquettes provenant d'un cas de thrombocythémie primitive et d'un cas de thrombocythémie secondaire été comparée à celle d'individus normaux. Dans les deux cas, on observe un grand polymorphisme des granulations denses forme en bâtonnet, en cocarde, en massue On rencontre également de nombreuses plaquettes contenant d'énormes vacuoles, et ceci tout particulièrement dans le cas de thrombocythémie primitive. Chez ce dernier le système des éléments clairs d origine golgienne est également très développé.

Summary

The ultrastructure of the blood platelets of two patients with respectively primary and secondary thrombocythaemia was compared with the platelets of normal control. In both cases, various drumstick-shaped, rod-shaped, and bulb eye-shaped granulations were observed. Many plat lets with large vacuoles were also found; they were especially prominent in the case of primary thrombocythaemia, where the amount of clear elements of the Golgi apparatus was increased.

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Extramedullary Hematopoietic Tumors of the Cranial Dura mater

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Extramedullary hematopoiesis is known to accompany a variety of hematological disorders and diseases of the bone marrow [15] This is usually encountered in a diffuse form, however occasionally it presents as tumor like masses in various sites [1 9] Reference to the cranial dura as a site of extramedullary hematopoiesis, however has rarely been recorded [3 6]

Recently we have encountered, incidentally at autopsy extra medullary hematopoietic masses of the cranial dura mater in 3 patients with different hematological disorders. These masses had not produced symptoms during life The rarity of the involved site justifies a description of the cases.

Case Reports

Case 1

A 9-year-old boy known to have suffered from thalassemia major since the age of 9 months was hospitalized on numerous occasions. Symptomatic treatment consisted of frequent blood transfusions and splenectomy was performed at the age of 3 years. This resulted in 6 months-period of remission but thereafter his condition deteriorated, skeletal changes appeared, and on occasions the hemoglobin dropped to 2 2.5 g Frequent hospitalization was necessitated by the need for blood transfusions or for episodes of fever or congestive heart failure

During his last admission for general weakness and pyrexia (40°C) uncontrollable diarrhea supervened and the patient died in comatous stat

Autopsy revealed child with generalized edema and 'thalassemic facies The bone marrow was markedly hyperplastic, particularly the ribs, the long bones and skull, with marked thickening of the diploe. There was hypertrophy and dilatation of the heart, cholelithiasis, generalized lymphadenopathy cirrhosis of the liver with secondary hemochromatosis and extensive hemosiderosis of the pancreas, thyroid, adrenals, lymph nodes and salivary glands. I both the bone marrow and lymph nodes aggregates of foam cells containing PAS positive material were found. There ere multiple recent hemorrhages in the lungs, subpleurally in the adrenal glands and the subarachnoid space.

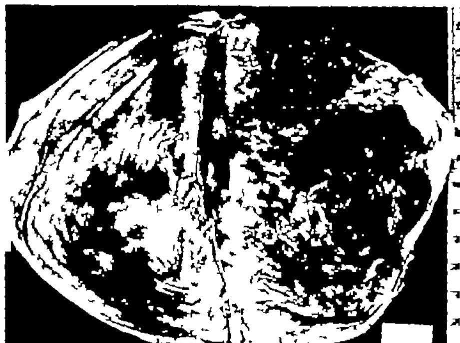


Fig 1 Case 1 Cranial dura mater showing multiple tumoral masses.

The left inner surface of the cranial dura mater was covered by soft confluent reddish tumor-like masses, measuring up to 2 cm in diameter which impressed the adjacent surface of the cerebral hemisphere (fig 1) Histological examination showed these masses to consist of hematopoetic tissue containing mature and immature elements of both the white and the red cell series (fig 2) The red blood cell precursors, however were predominant. Mature lymphocytes, plasma cells and megakaryocytes were also present.

Case 2

A 68-year-old male was repeatedly hospitalized for hematological disorder of 3 years duration characterized by pancytopenia, and hyperplastic bone marrow In the course of the disease recurrent severe gastro-intestinal bleeding occurred but the precise origin of the bleeding could not be defined. During one of these admissions to hospital, year before his death, following an episode of massive gastro-intestinal bleeding and vigorous blood transfusion the peripheral blood picture had altered. There was now leucocytosis of 20,000 per mm³ with 40% immature white blood cells. Immature red blood cells were also demonstrated.

Because of negative leucocyte alkaline phosphatase reaction, markedly increased serum vitamin B_{12} level of 1,200 µµg/ml and the presence of the Philadelphia chromosome the diagnosis of chronic myeloid leukemia was established.

The patient received blood transfusions and corticosteroids but 3 weeks prior to death, the course of the disease was complicated by an acute attack of biliary colic due to choledocholithiasis. He underwent operation and cholecystectomy was performed, however the patient died suddenly after the operation with the clinical picture of shock.

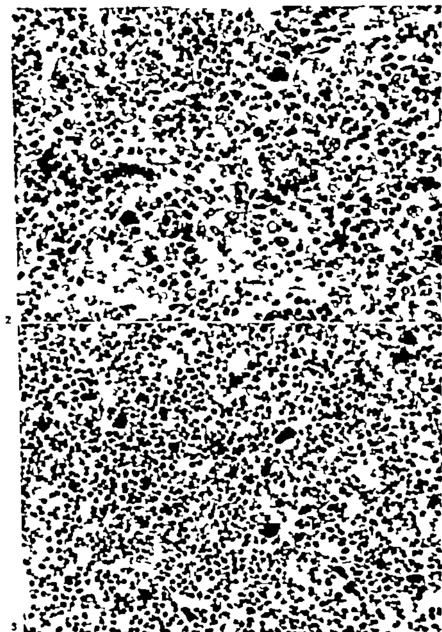


Fig. 2. Case 1. Histological section from one of the masses shown in figure 1 demonstrating hematopoietic tissue containing cells of both the myeloid and erythroblastic series and megakaryocytes. Hematoxylin and eosin. × 260.

Fig. 3. Case 2. Histological section from dural hematopoietic masses. Hematoxylin and eosin; × 260.

Autopsy revealed an extensive hemoperitoneum. There was dilatation and hypertrophy of the heart with pulmonary edema. The bone marrow was hyperplastic and composed mainly of white blood cell precursors. There was extensive extramedullary hematopoiesis in the liver, lymph nodes, spleen, kidneys, perinephric and peripelvic fat.

Scattered throughout the cranial dura mater and on the inner surface of the falx were numerous soft, irregular red tumor-like masses measuring up to 3 cm in diameter. Histological examination revealed these to be composed of hematopoietic tissue (fig. 3).

Case 3

A 48-year-old male suffering from myelofibrosis since 1953 was hospitalized a number of times during the period 1953 to 1957. In 1955 there was marked hepatosplenomegaly due to myeloid metaplasia, thereafter polycythemia developed and jaundice appeared. He was treated with myleran and irradiation to the spleen, however pancytopenia developed as a complication of myleran treatment, and the use of this drug was stopped. In October 1957 splenectomy was performed. The spleen was found to be enlarged, weighed kg, and showed extensive areas of extramedullary hematopoiesis and hemosiderosis. Seven months later portal hypertension with recurrent ascites developed, but local irradiation to the liver resulted in reduction in its size. In November 1957 he was hospitalized again because of bleeding tendency which presented as epistaxis, gingival and conjunctival bleeding and rapidly progressing glaucoma due to retrobulbar hemorrhage. Examination revealed severe jaundice, marked hepatomegaly and ascites. There was anemia, 7.6 g Hb, [illegible]9 million erythrocytes, thrombocytopenia 24 000–80 000. The nucleated cell count ranged from 84 to 290,000/mm³ with 50% erythroblasts. During the course of hospitalization there was gradual deterioration in his condition and he died after prolonged restlessness with terminal clouding of consciousness.

At *autopsy* there was jaundice of the mucous and serous surfaces and petechial hemorrhages were found in the skin, conjunctiva, and mucosa of the gastrointestinal tract. Digested blood was present in the stomach. The bone marrow showed areas of myelofibrosis, scattered cellular areas of active hematopoiesis and a number of foci compatible with myeloid leukemia. The liver weighed 3,070 g and there was marked inguinal, paraaortic, cervical and iliac lymphadenopathy. Histology revealed extensive diffuse extramedullary hematopoiesis in these organs and in the kidneys. In addition there were well demarcated areas of soft reddish-brown tumor-like tissue surrounding the dilated portal veins in the liver, in the peri-renal and peri-adrenal fat tissue, in the mesentery of the intestines, and in the kidney. Histologically these proved to be masses of extramedullary hematopoietic tissue. Diffuse petechial and ecchymotic subarachnoid, and intracerebral and intramedullary hemorrhages were also found.

The inner aspect of the cranial dura mater revealed a number of irregular soft nodules of reddish-brown tumor-like tissue, varying in size up to 2 cm in diameter (fig. 4). Histologically these proved to be masses of extramedullary hematopoiesis with a relative paucity of megakaryocytes.

Discussion

Many of the cases with extramedullary hematopoiesis reported in the literature have shown the presence of nodular tumor-like masses of hematopoietic tissue in a variety of situations. These sites include the pleura, mediastinum, peritoneal surfaces, the perirenal connective tissue, mesentery, lymph nodes, liver and spleen [1 9 11 12, 13]. Occasional cases have been recorded involving the anus [5] and testis [8]



Fig. 4. Case 3. Cranial dura mater showing number of tumoral masses.

Few cases have been reported involving the dura mater of the spinal canal and causing spinal cord compression [2, 4, 10, 14]. In reviewing the literature we have found involvement of the cranial dura recorded on only 2 instances. In 1927 BRANNAN [3] described a nodular tumor mass in the cerebral falx of a 7 1/2-month-old infant with von Jaksch's anemia. In 1930 HU and CASH [6] described 2 cases with extramedullary hematopoiesis as a complication of Kala Azar resulting in the formation of extradural tumors. The tumor-like masses, consisting of hemopoietic elements, in the cases reported here, developed during the course of chronic leukemia, myelofibrosis with terminal leukemia and thalassemia; however, their presence in the dura overlying the brain was not accompanied by any symptoms during life. We have no ready explanation for the prominence of the rare dural involvement in our cases. However, it is interesting to record that splenectomy had been performed prior to death in 2 of the 3 cases described. This may well be coincidental, but it is possible that this procedure may influence the development of extramedullary hematopoietic foci in unusual sites,

such as the cranial dura, as encountered in 2 of the cases reported above

Summary

Three examples of extramedullary hematopoietic tumors of the cranial dura mater are described as incidental findings at autopsy. These rare dural tumors were encountered in 3 different patients with thalassemia major, chronic myeloid leukemia and myelofibrosis with terminal leukemia, and were not accompanied by any clinical symptoms during life.

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Medizinische Universitätsklinik Innsbruck (Vorstand Prof. Dr. H. Braunsteiner)

Der immunzytologische Lysozymnachweis in menschlichen Blutzellen

H. Asamer, F. Schmalzl und H. Braunsteiner

Der Nachweis von vermehrten Lysozymmengen im Urin von Patienten mit Monozytenleukämie [1] hatte zahlreiche Untersuchungen über die diagnostische Bedeutung unterschiedlichen Serum- und Uringehaltes bei hämatologischen Erkrankungen zur Folge [2, 3, 4]. Bisher standen allerdings nur indirekte Methoden zur Bestimmung des Lysozymgehaltes von Blutzellen zur Verfügung. Die Messung von Lysehöfen um Blutzellen in Micrococcus-Lysodeikticus-Suspensionen [5] erfaßt nur das freigesetzte Lysozym, die Fermentbestimmung in Blutzellzytolysaten [2, 4] ist lediglich bei einheitlicher Zellpopulation von Nutzen. Es wurde daher die immunhistologische Methode zum intrazellulären Lysozymnachweis in peripheren Blutausstrichen herangezogen [6]. Im folgenden werden die immunzytologische Technik, die Speziesspezifität von Lysozym und Ergebnisse mit der quantitativen Mancini-Technik beschrieben.

Material und Methode

1. *Anti-human-lysozym-serum (AHL).* Zwei Kaninchen wurden mit insgesamt je 6 mg Humanlysozym in komplettem Freundschem Adjuvans immunisiert und nach 6 Wochen entblutet. Nach Absorption mit Human-γ-G-Globulin [1] zeigte sich mit der Ouchterlony-Technik gegen Humanlysozym eine einzige scharfe Präzipitationslinie (Abb. 1).

Die 2%ige Ammoniumsulfatfraktion wurde im Verhältnis 30 mg FITC (Fa. Hyland) pro g Eiweiß ohne Acetonzusatz 16 h unter ständigem Rühren bei 4°C gekoppelt und nach Sephadex-G-25-Filtration 1%ig verwendet [7]. Teilweise wurde diesem Konjugat unmittelbar vor Gebrauch im Verhältnis 1:1 an Rhodamin gekoppeltes 5%iges Bovinalbumin (RHB) zugesetzt.

Anti-egg-lysozym-serum (AEL). Es wurde wie oben mit insgesamt 6 mg Lysozym (von Hühnereiweiß) Lot 72294 Calbiochem, gewonnen und an FITC gekoppelt.

Wir danken Herrn Dr. E. F. Osserman, New York, für die Überlassung von gereinigtem Human-Lysozym.

Blutausstriche. Luftgetrocknete periphere Blutausstriche wurden 5 min in 95% Äthanol fixiert und nach erneuter Lufttrocknung gründlich in Coonspuffer pH 7,2 gewaschen. Anschließend wurde 30 min in einer feuchten Kammer bei Zimmertemperatur mit einem Tropfen fluoreszierendem Antiserum inkubiert, dann erneut in Coonspuffer gewaschen und mit Gurr Univert Mountant Aqueous eingebettet.

Ouchterlony-Technik. Verwendet wurde Special Noble Agar (Difco Laboratories Detroit) 2% in Veronalpuffer pH 8,2, Ionenkonzentration 0,1.

Mancini-Technik. Der für die Ouchterlony-Technik verwendete Agar wurde im Wasserbad kurz aufgekocht und nach Abkühlung auf 50°C 10% mit AHL versetzt. 2,5 ml dieses erwärmten Gemisches wurde sodann auf einen Objektträger der Größe 7,6 mal 2,6 plan ausgegossen. Nach dem Erstarren des Agars wurden Hohlzylinder von 3 mm Durchmesser ausgestanzt und mit einer Mikropipette 0,002 ml der Probe eingepipettiert. Weitere Methodik siehe [8].

Mikroskop. Zetopan der Firma Reichert mit Fluoreszenzausrüstung und Photoautomatik.

Ergebnisse

Ouchterlony-Technik. In der Rosette 1 (Abb. 1a) wurde in der Mitte das zur Immunhistologie verwendete AHL aufgetragen, in den peripheren Hohlzylindern bei 12 Uhr beginnend, im Uhrzeigersinn Humanlysozym 1% in geometrischer Verdünnung bis 1/32. Rosette 2 (Abb. 1b) in der Mitte AHL wie oben, bei 12 Uhr Hühnereiweißlysozym, dann Serum der Patientin Mü. mit chronischer myeloischer Leukämie, weiters Humanlysozym 1%, Serum der Patientin Pi. mit zytochemisch verifizierter [9] Monozytenleukämie, menschliche Tränenflüssigkeit in Originalkonzentration und nochmals Humanlysozym 1%. Die menschlichen Lysozyme verschiedener Herkunft zeigen eine immunologische Identität [1, 3], hingegen besteht keine Kreuzantigenität gegenüber Hühnereiweißlysozym. Semiquantitativ lassen sich im Serum der Patientin Pi., nicht aber in dem der Patientin Mü., geringe Lysozymmengen nachweisen, deutlich mehr in menschlicher Tränenflüssigkeit.

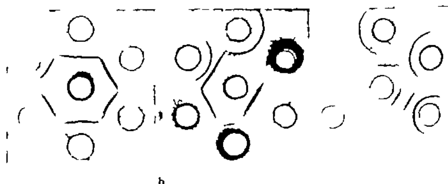


Abb. 1 Untersuchungen von Antihumanlysozym mit der Ouchterlony-Technik. Erklärung siehe Text

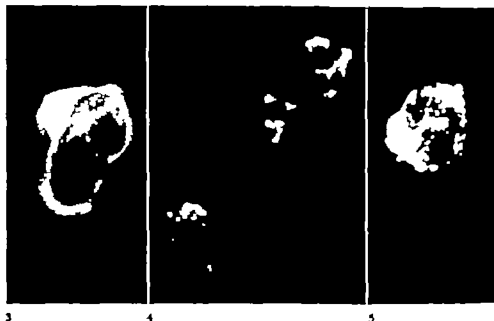


Abb. 2. Untersuchung von Serum und Urin einer Patientin mit Monozytenleukämie mit der Mancini-Technik. Erklärung siehe Text. Die angedeuteten Präzipitathöfe sind durch den γ-Globulingehalt der Humanseren und des eingeengten Urins verursacht, da hier unabsorbiertes AHL verwendet wurde

Rosette 3 (Abb. 1c): in je 2 benachbarte periphere Hohlzylinder wurden Hühnereiweißlysozym und Humanlysozym 1 beginnend, weiter 0,1 und 0,05, in der Mitte AEL aufgetragen. Es zeigt sich auch hier keine Kreuzreaktion zwischen diesen beiden Lysozymen.

Mancini-Technik (Abb. 2): Es wurden von links nach rechts jeweils 0,002 ml des Serums der Patientin Pi., des Serums der Patientin Mü., 50fach eingeengter Urin der Patientin Pi. und Hühnereiweißlysozym einpipettiert. Es zeigt sich, daß lediglich der Urin der Patientin Pi. einen meßbaren Präzipitationshof bildet (bei Serum Pi. angedeutet), der bezogen auf die mit Humanlysozym erstellte Eichkurve einer Menge von 180 mg entspricht. Da diese Urinprobe auf das 50fache eingeengt war, können für die 24-Stunden-Ausscheidungsmenge von 770 ml 36 µg/ml errechnet werden.

Immunzytologische Untersuchungen: In peripheren Blutausstrichen zeigten lediglich Granulozyten und etwas schwächer auch Monozyten mit AHL eine zytoplasmatische Fluoreszenz (Abb. 3 und 4). Lymphozyten, Erythrozyten und Thrombozyten waren negativ. Bei Verwendung von fluoreszierendem AEL fiel eine deutliche Fluoreszenz eines Teiles der Granulozyten bei ansonsten negativem Befund auf (Abb. 5). Bei Inkubation einer aa Mischung von AEL und RHB fluoreszierten jene Zellen rötlich und konnten als eosinophile Granulozyten identifiziert werden. Wurden die Ausstriche mit einem AHL-RHB-Gemisch inkubiert, war die zytoplasmatische Fluoreszenz der neutrophilen Granulozyten spezifisch apfelgrün, während die eosinophilen das rötlich fluoreszierende Rhodamin unspezifisch adsorbierten. Ausstriche von peripherem Hühnerblut waren mit AHL komplett negativ, während mit fluoreszierendem AEL Granulozyten und Mono-



3 4 5

Abb. 3. Ausgeprägt spezifische Fluoreszenz in einem Monozyten (Normalperson, peripheres Blut) nach Inkubation mit fluoreszierendem Antihumanlysozym (×1600)

Abb. 4. Zwei neutrophile Granulozyten und ein Monozyt aus dem peripheren Blut einer Normalperson. Spezifische Fluoreszenz nach Inkubation mit fluoreszierendem Antihumanlysozym (800)

Abb. 5. Eosinophiler und neutrophiler (links) Granulozyt. Peripheres Blut einer Normalperson. Inkubation mit fluoreszierendem Antihühnereiweißlysozym (AEL). Der neutrophile Granulozyt zeigt keine Reaktion infolge mangelnder Kreuzantigenität zwischen Human- und Hühnereiweiß-Lysozym, ebenfalls keine unspezifische Adsorption. Dagegen tritt in dem eosinophilen Granulozyten eine ausgeprägte granuläre Fluoreszenz durch unspezifische Adsorption von AEL auf (800).

zyten nach Art der analogen humanen Blutzellen fluoreszierten. In peripheren Blutausstrichen von 2 Patienten mit chronischer lymphatischer und einem mit akuter myeloischer Leukämie war in den Leukämiezellen kein Lysozym bei 2 Patienten mit chronischer myeloischer Leukämie nur sehr wenig nachzuweisen. Die monozytären Zellen der Patientin Pi. zeigten eine deutliche spezifische, zytoplasmatische Fluoreszenz.

Diskussion

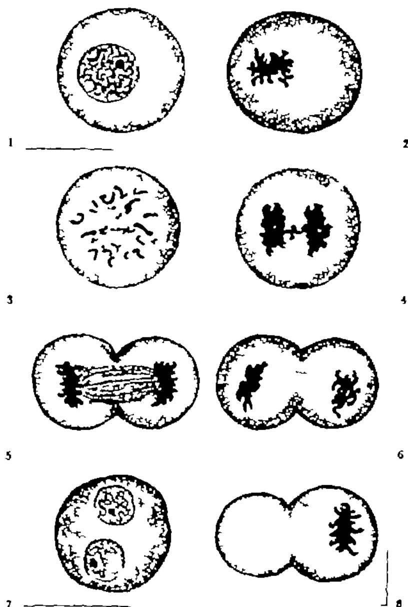
Mit der Ouchterlony Technik konnte eine Identitätsreaktion menschlicher Lysozyme verschiedener Herkunft gezeigt werden zwischen

Humanlysozym und Hühnereiweisslysozym besteht hingegen keine Kreuzantigenität. Dies steht im Einklang mit Befunden von OSSERMAN [1] der mit Antihumanlysozym lediglich die lytische Aktivität von Humanlysozym nicht aber die von Hühnereiweisslysozym hemmen konnte. Weiterhin sprechen auch Aminosäureanalysen für Strukturunterschiede dieser beiden Lysozyme [1 3 9] Die fehlende Kreuzantigenität bestätigt die Spezifität des immunohistologischen Lysozymnachweises, da dieser nur mit homologen fluoreszierenden Antiseren gelang Semiquantitativ konnte mit der Ouchterlony Technik der hohe Lysozymgehalt menschlicher Tränenflüssigkeit bestätigt werden.

Mit der quantitativen Mancini Technik liess sich bei einer Patientin mit Monozytenleukämie eine Urinlysozymkonzentration von 36 μg/ml errechnen. OSSERMAN [1] fand mit einer enzymatischen Methode bei 10 Patienten mit monozytärer bzw monomyelozytärer Leukämie im Urin Lysozymkonzentrationen zwischen 25 und 420 μg/ml. Bei 270 weiteren untersuchten Fällen mit verschiedensten Erkrankungen einschliesslich anderen Leukämieformen lagen die Durchschnittswerte zwischen 3–5 μg/ml lediglich bei manchen Patienten mit chronischen Entzündungen kamen Überschneidungen mit niedrigen Urinlysozymkonzentrationen von Monozytenleukämien vor In unserem Fall handelte es sich um eine chronische Form, die ausserdem bereits zytostatisch behandelt war so dass die relativ niedrige Lysozymmenge erklärbar ist.

Erhöhte Serumlysozymkonzentrationen werden bei manchen myeloischen Leukämien und bei Monozytenleukämien erniedrigte bei lymphatischen Leukämien beschrieben [2 4] Während darüber einheitliche Befunde vorliegen, sind die Angaben über den Lysozymgehalt von Leukämiezellen bei den verschiedensten Formen der myeloischen Leukämie widersprüchlich. So finden NOBLE *et al* [2] in Zellzytolysaten bei der chronischen myeloischen Leukämie normale bei der akuten myeloischen in Abhängigkeit von der Blastenzahl erniedrigte Werte

PERILLIE *et al.* [4] beschreiben die grösste Lysozymaktivität in Leukozyten von Patienten mit chronischer myeloischer Leukämie eine erhöhte auch bei Myeloblastenleukämie. Der intrazelluläre Lysozymnachweis orientiert in der hier angegebenen Form rasch über den Gehalt dieses Fermentes in peripheren Blutzellen und ermöglicht damit auch eine exaktere Beziehung zwischen Art und Anzahl lysozymbildender Blutzellen und Serumlysozymspiegel aufzuzeigen.



Figs. 1–8. The drawings illustrat schematically the types of chromosomal abnormalities. *(1) Erythroblasts from control embryo howing central nucleus, with nucleolus* and very fine chromosomal pattern. *(2)* Erythroblast from the embryo irradiated with 230 Note the striking lumping and sticking together of chromosomes. *(3)* Erythroblast from control embryo showing normal metaphase chromosomes. *(4)* Anaphase figure with bridge formation and berrant chromosomes in an erythroblast from the subjected to the combined

Table I. Percentage distribution of mitotic phases

Groups	Average percentage of cells in various mitotic stages		
	Prophase	Metaphase	Ana-telophase
Control	46	30	24
Urethane	44	29	27
X-rays	32	25	43
Urethane followed by X-rays	27	52	21

P<0.01
P<0.001

Table II. Mitotic indices of various mitotic stages

Group	Prophase	Metaphase	Ana-telophase	Mean mitotic index
Control	2.09	1.306	1.09	1 4986
Urethane	2.310	1.526	1 421	1 7523
X-rays	2.462	1.923	3.301	2.5616
Urethane followed by X-rays	3.856	7 428	3.00	4 7613

Treatment with urethane followed by X-radiation. In another series (III) the unincubated eggs injected with urethane as bove were incubated until the definitive primitive streak stage. They were then irradiated with 250 and incubated for another 82 h to obtain 4 day old embryos. An equal number of untreated embryos incubated for 96 h served as controls.

Smear technique. Blood was removed from the heart of embryos with fine capillary pipette. Smears were prepared by usual technique. The air-dried smears were then fixed and stained in May-Grünwald-Giemsa stain.

Chromosome analysis. Two scoring procedures were used. I the first procedure erythroblasts showing inhibition of mitosis at various phases were noted. Counting was made and percentage distribution of mitotic phases (i.e. percentage of any single mitotic phase in 100 mitoses) was recorded in table I. In the second procedure, more detailed structural abnormalities were scored. Cells presumably containing structural anomalies were selected

treatment with urethane and X-rays. *(5)* Anaphase figure showing the spindle fibres and 'clumped' chromosomes in an erythroblast from the embryo irradiated with 250 *(6)* 'Binucleate' erythroblast from the embryo irradiated with 250 showing an incomplete membrane *(7)* A 'true binucleate cell from the embryo subjected to the combined treatment with urethane and X-rays. *(8)* Erythroblast, in which the chromosomes of one daughter nucleus disintegrated. This embryo was subjected to the combined treatment of urethane and X-ray

from the survey of first procedure. Drawings were made of selected cells under camera lucida and anomalies were recorded.

Results

Table I shows the percentage distribution of mitotic phases in the embryonic erythroblasts. Counts made on an equal number of blood cells of the untreated specimnes of the same age are given for comparison It is evident that after treatment with X ray alone, there is a relative decrease in the percentage of cells in prophase and a distinct rise in the percentage in anaphase and telophase ($P < 0.01$) whereas the percentage of cells in metaphase is not significantly altered.

After the combined treatment with urethane and X-rays, there is a considerable decrease in the percentage of prophases and a distinct rise in the percentage of cells in metaphase ($P < 0.001$) The percentage of cells in later phases (ana-telophase) is not different from normal controls.

Table II shows the mean mitotic index as well as the indices of the individual mitotic phases in the erythroblasts of (1) untreated (control) embryos, (2) those treated with X-irradiation alone and also of (3) embryos treated with urethane followed by X-rays. These findings show that the mitotic index reaches a peak after the combined treatment with X ray and urethane. Thus distinct rise of mitotic index is caused by a large increase of cells in metaphase and a general rise of cells in other mitotic phases. The increase in the mitotic index after X radiation alone (2.5616) results from an equal increase of all phase indices.

Distinct morphological changes were observed in erythroblasts during the course of mitosis both in the specimens treated with X-ray alone or with urethane followed by X rays. Clumping and sticking together of the chromosomes were the striking effects (fig 2) The most frequent changes were found in the anaphase stage and consisted in chromosomal bridges (fig 4) and a-centric fragments. In a few cases the spindle mechanism was incomplete or absent.

Binucleate cells were common after the treatment. They showed an incomplete membrane between two potential cells (fig 6) or as was more frequent, the membrane was absent and the cell can be described as a true binucleate cell (fig 7) Instances in which the chromosomes of one daughter nucleus disintegrated during mitosis were also found (fig 8)

Discussion

It is now well established that ionizing radiations inhibit mitosis and induce chromosomal aberrations in mammalian cells [1 9] The lymphatic tissues are particularly susceptible [10 11] The present data also show that the administration of 250 r to chick embryos induces mitotic inhibition and chromosomal aberrations in the erythroblasts.

Urethane is a radiomimetic substance able to produce mutations. Like irradiation it inhibits mitosis and induces chromosomal aberrations in mammalian cells [6 7] and chick erythroblasts [13] Furthermore, urethane-induced mitotic inhibition and structural aberrations are recovered partially in chick erythroblasts [13] a well as in mammalian cells [12] by subsequent treatment with thymine. These studies indicate that urethane induced mitotic inhibition and chromosomal aberrations might primarily be due to the inhibitory effects of the substance on cellular DNA.

Enhancement of radiation effects in both normal and neoplastic tissues has been reported when urethane is administered either before or after X-ray treatment [14 15] Synergism of urethane and X-rays has also been demonstrated in chick embryos [16] From the results of the present investigation it seems that while X-rays cause a rise in the mitotic index, due to a general rise in various phase-indices (table II) the mitotic index is significantly lower than that observed after combined treatment of urethane and X rays. The percentage distribution of mitotic phases after the combined treatment is also remarkably different from that found after administration of X-rays alone (table II) There is a distinct rise in the percentage of cells in metaphase after the combined treatment when compared to that of X-rays alone These results suggest that there is an acceleration of the prophase stage and a prolongation of later phases when urethane is added before the X ray treatment. MOELLENDORFF [17] and BUCHER [18] observed a similar rise in the percentage of metaphase on fibroblasts growing *in vitro* after treatment with urethane

The exact mechanism responsible for the radiation-induced mitotic inhibition is not yet known In an interesting paper on the enhancement of radiation induced mitotic inhibition by the incorporation of thymine analogue (BudR) in L. cells, SCHNEIDER and JONES [19] have demonstrated that DNA is involved in some way in the radiation induced inhibition The inhibition of DNA synthesis by urethane appears to result from the inhibition at the site of thymine biosynthesis

[20 21] Boyland [22] has suggested that urethane prevents the methylation of uracil to thymine. These effects of urethane associated with thymine of the DNA might be additive with the changes in DNA produced by irradiation. The exact site and modes of interaction be tween urethane and X rays still remains obscure.

Summary

I chick erythroblasts X-rays increase the mitotic index. This is due to more or less equal rise of the indices of all phases. The combined treatment with urethane and X-rays produced a distinct rise in the mitotic index with significant increase of the cells in metaphase, which may be due to an acceleration of the prophase and prolongation of later phases (anaphase and telophase). The significance of these observations is discussed and the possible site of interaction between the two agents is suggested.

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Libri

A. Fieschi, G. Sacchetti: **Aspetti di fisiopatologia e clinica dell'ipersplenismo.** Pozzi, Roma 1967. 336 p.

This monograph summarizes the authors' own experience with respect to various hematologic disorders related to so-called hypersplenism. The rate of destruction of blood cells, splenic pooling and the relationships between spleen and bone marrow function were carefully evaluated. The methods employed are of general clinical interest. The available literature on 'hypersplenic syndromes' is adequately reviewed. The overall presentation of clinical data is somewhat lengthy. No revolutionary conclusions can be drawn from the available data, but the book is a useful source of information to clinicians and clinical pathologists interested in various hematologic abnormalities related to disorders of the spleen.

E. Beck, Basel

R. J. Hoagland: **Infectious Mononucleosis.** Grune & Stratton, New York 1967. 152 p. Price: US-$ 7.50.

It is always refreshing to see a book in which the author express his personal opinions who differ from the commonly accepted ones. The book by Hoagland is a case in point. It claims that many of the concepts and observations published so far are erroneous. The book is based on personal experience on 500 consecutive cases of i.m. Undoubtedly many of Hoagland's conclusions and ideas will be sharply contested by other experts in the field, e.g. if he states that as 'a personal conviction rather than established facts' he doubts, that the causative agent of i.m. is of a viral nature. The most likely nonviral cause is in his opinion a protozoan. He believes that i.m. does not occur in epidemic form. The chapter on pathology and pathogenesis is mainly a survey of the respective literature (p. 10–21). In a short historical background (p. 3) Hoagland disclaims among other statements that 'Pfeiffer's Drüsenfieber' was infectious mononucleosis.

In his concluding remarks he states as one of *his objectives in writing this book is to* rectify erroneous impressions. In correcting errors one must sometimes be iconoclastic.

G. Rosenow, New York, N.Y.

L. D. Leder: **Der Blutmonocyt.** Springer, Berlin 1967. 293 p., 73 fig.

In der vorliegenden Monographie gibt der Autor einen eingehenden Überblick über das umfangreiche Material, das über Herkunft und Funktion der Monocyten zusammengetragen wurde. Der Besprechung eigener ausführlicher Arbeiten zur Frage der Herkunft dieser Blutzellen sowie eigener Untersuchungen mit der Rebuckschen Hautfenstertechnik wird grosser Raum gegeben. Der Autor gelangt auf Grund zytochemischer Untersuchungen zu dem Ergebnis, dass die Blutmonocyten aus dem Knochenmark stammen und sich dort aus Promyelocyten entwickeln. Es handelt sich hierbei um offensichtlich reife Promyelocyten, die durch einen sehr hohen Gehalt an Naphthol-AS-D-Chloracetat-Esterase und Peroxydase gekennzeichnet sind. Mit ausgezeichneten Abbildungen sucht der Autor seine Theorie zu belegen. Während die myelogene Herkunft der Monocyten tatsächlich als weitgehend *gesichert angesehen werden darf, sind den gelieferten Beweisen* für die Entwicklung der Monocyten aus den dargestellten reifen Promyelocyten einige Vorbehalt entgegenzubringen. Sehr zu Recht betont der Autor die zentrale Stellung der Monocyten im Ablauf der lokalen Entzündungsvorgänge und weist auf ihre Bedeutung für die Entstehung von Makrophagen hin. Die Monocytenleukämie ist als zytochemisch eindeutig identifizierbare Myelose aufzufassen, und die Unterscheidung verschiedener Typen dieser Leukämie wird abgelehnt. Einige praktische Hinweise vervollständigen dieses zweifellos wertvolle Buch, das für jeden, der sich mit der Problematik der Blutmonocyten befasst, von grösstem Nutzen ist. Besonders hervorzuheben sind die reichhaltige Bibliographie und die vorzüglichen Abbildungen.

F. Schmalzl, *Innsbruck*

Das Internationale Nomenklaturkomitee für Hämatologie

Bericht über die bisherige Tätigkeit

Das „Expert Panel on Hematological Terminology of the International Committee for Standardization in Hematology“ ist zugleich das „Nomenclature and Glossary Committee of the International Society of Hematology“ und wurde im Frühjahr 1965 ins Leben gerufen. Bei der Zusammensetzung des Komitees wurde darauf geachtet, dass bei einer echten Internationalität alle Sparten der Hämatologie und auch von einander abweichende Meinungen und Schulen vertreten seien. Das Komitee setzt sich wie folgt zusammen:

Vorsitzender: Heilmeyer (Ulm). Sekretär: Boroviczény (Freiburg i. Br.). Mitglieder: Alexeieff (Moscow), Astaldi (Tortona), Barkhan (London), Bart (Pécs), Bernard (Paris), von Bonsdorff (Helsinki), Braunsteiner (Innsbruck), Brinkhous (Chapel Hill, N.C.), Dameshek (New York, N.Y.), Fieschi (Genova), Gouttas (Athen), Hauptmann (Zagreb), Hellemann (Utrecht), Hittmair (Innsbruck), Künzel (Berlin), Libansky (Prag), Lortholary (Paris), Mancilla (Genève), Mayer (Strasbourg), Osgood (Portland, Ore.), Palos (Budapest), Plum (Dänemark), Rosenfeld (Sao Paolo), Sansonnens (Genève), Seegers (Detroit, Mich.), Stohlman (Washington, D.C.), Tempka (Warszawa), Trincão (Lisboa), Undritz (Basel), Verloop (Utrecht), Watanabe (Hiroshima), Wintrobe (Salt Lake City, Ut.), Wright (New York, N.Y.). Philologischer Berater: Erren (Freiburg i. Br.), Berater für Dezimalklassifikation: Nacke (Bielefeld). Lexikographische Berater: Mancilla (Genève) und Nicole (Genève). Zoohämatologie: Andrew (Indianapolis).

Das Sekretariat dieses Nomenklaturkomitees wurde im „Institut für Standardisierung und Dokumentation im Med. Laboratorium“, Freiburg i. Br. eingerichtet. Eine an dieses Institut gegebene finanzielle Unterstützung der World Health Organization ermöglichte Anfang 1966 die Aufnahme der Arbeit. Komiteemitglieder wie auch andere Experten erhielten die Aufforderung, einen geeigneten Terminus technicus für einen Begriff vorzuschlagen und dazu eine Definition auszuarbeiten. Diese Vorschläge wurden nach einer redaktionellen Bearbeitung zuerst von der Gruppe der „Chief Reviewers“ (Bernard, Dameshek, Heilmeyer, Hittmair, Undritz und Wintrobe) diskutiert, anschliessend von der Übersetzergruppe (Alexeieff, Boroviczény, Guasch, Mayer und Erren) erneut diskutiert und übersetzt. Es wurde ein griechisch-lateinischer internationaler Terminus festgesetzt, seine Ethymologie erläutert und dieser in das Englische, Deutsche, Französische, Spanische und Russische übertragen. Wo es notwendig erschien, wurde eine Kurzform festgestellt oder auch eine Erläuterung zur eigentlichen Definition gegeben. Synonyme wurden aufgezählt, das Antonym genannt. Soweit bekannt, wurde die Literaturstelle, in der der Terminus zuerst vorkommt, genannt. Die Dezimal-Klassifikation-Zahl wurde festgelegt. Nach Erledigung dieser Arbeiten wurde das Manuskript zur Diskussion allen Komiteemitgliedern zugeleitet.

Es hat sich als nützlich gezeigt, nicht nur einzelne Begriffe, sondern auch in der hämatologischen Nomenklatur häufig vorkommende Wortelemente festzulegen und zu definieren. Bis jetzt konnte das Komitee Manuskripte über die folgenden Wortelemente und Termini verabschieden: -aemia, aniso-, -chrom, -chromasia, coagul( )-, -crit, -cytosis, dys-, eosinophil( )-, erythr( )-, haem-, hyper-, hypo-, karyo-, leuk( )-, lymph( )-, macro-, megakaryo-, mast(o)-, met-, micro-, myelo-, neutrophil( )-, oligo-, pachy-, para-, -penia, plasma-, plasmo-, pleio-, photo-, plastul( )-, -poiesis, poikilo-, poly-, pro-cyt, -rrhexis, sero-, schisto-, thromb( )-; Anaemia; Anaemia dyserythropoietica hereditaria; Anaemia ex haemorrhagia; Anaemia hyperchromica; Autoagglutinine; Basophilocyt; (Vasa) Capillaria (sanguinis); Corpusculum sanguinis; (Vasa) Capillaria venosa; Chlorocruorin; Coproporphyria erythropoietica; Cruor; Cruorin; Corpusculum Döhle; Eosinophilocyt; Erythro-

cuprein Erythrocytometria Fragilitas erythrocytorum Haem Haematin; Haematocrit Haematologia Haemocyanin Haemerythrin Hypersegmentatio hereditaria (Undritz) Hyposegmentatio hereditaria (Pelger-Huët); Lympha; Lymphocyt Macula Schüffneri Monocyt, Myoglobin Neutrophilocyt Plasma sanguinis Plasmocyt Porphyria, Porphyrin Protoporphyria erythropoietica Sanguinocyt Sprue Sanguis Serum sanguinis, Testis antiglobulini (Coombs); Verdoglobin Volumen cellulae pactae.

Dieses Material ist bereits der Vollversammlung des International Committee for Standardization in Hematology (ICSH) übergeben worden. Die endgültige Abstimmung darüber wird im kommenden Jahr brieflich erfolgen. Das Komitee konnte in der Zwischenzeit durch die Bildung von einzelnen Arbeitsgruppen und Subkomitees, durch Zusammenarbeit mit anderen internationalen Nomenklaturkomitees, mit der National Library of Medicine (Washington, D.C.) und Autoren von medizinischen Enzyklopädien, eine Anzahl weiterer Definitionen zusammenstellen, die sich zur Zeit noch in Diskussionen befinden. Danach soll das ganze Material veröffentlicht werden. Es ist geplant, in den kommenden Jahren die Arbeit weiterzuführen, bis die gesamte Hämatologie bearbeitet ist. Vorläufig kann das Sekretariat des Komitees nur in begründeten Einzelfällen und dann auch nur über jeweils einzelne Termini Auskunft erteilen.

Freiburg, November 1968 Dr. K. G. v. Boroviczény, Sekretär des Komitees

International Conference on Cryoprecipitated Factor VIII

An international conference on the preparation and use of cryoprecipitated factor VIII in haemophilia was held in Brussels, June 23rd, 1967 under the auspices of the Belgian Association for the Study of Haemophilia. The proceedings of this conference including the discussion was published. Orders for copies of this booklet are to be forwarded to the Office of the Belgian Society of Haemophilia, Minderbroedersstraat 29, *Leuven*, Belgium (2 US dollars, 110 pages). The benefits made are being donated to the World Federation of Haemophilia.

International Society on Toxicology

The 2nd International Symposium on Animal Toxins of the International Society on Toxicology will be held in Tel Aviv, Israel, February 22nd to March 1st, 1970.

Information on the scientific program: Prof. Andrée de Vries, POB 85, Petah Tikva, Israel.

Information on travel, hotels and excursions: Kenes, 30 Dizengoff St., Tel Aviv, Israel.

International Primatological Society

The 3rd International Congress of Primatology will be held in Zurich (Switzerland), August 3–5 1970. The Anthropological Institute of the University of Zurich has been appointed the organizer of this congress. Please address all inquiries to the secretary's office: Anthropological Institute, Künstlergasse 15, *CH-8001 Zurich*.

Acta haemat. 41 65–73 (1969)

Department of Pathology University of Cambridge and Department of Experimental Pathology and Cancer Research, University of Leeds

Arrest of Cell Proliferation and Protein Synthesis in Megaloblasts of Pernicious Anaemia

S N Wickramasinghe, D G Chalmers and E. H. Cooper

The combination of cytochemical and autoradiographic methods for the study of human erythroblasts has demonstrated an abnormality in the distribution of the megaloblasts of pernicious anaemia in interphase [18, 19] The main features of our findings were, firstly a decrease in the ratio of the number of megaloblasts synthesising DNA (S) to the number in the period of interphase (G_2) between the completion of DNA synthesis and mitosis (S/G_2 ratio) secondly the presence of several megaloblasts with DNA contents between the 2c (diploid) and 4c (tetraploid) modes which were not synthesising DNA as indicated by their failure to incorporate tritiated thymidine (^{3}H TdR) *in vitro* and thirdly that these defects were most pronounced in the early polychromatic megaloblasts. Some of these findings have been independently confirmed by Menzies *et al.* [9] and Yoshida *et al.* [20] Recently Wickramasinghe and Chalmers [17] have shown that in pernicious anaemia a significant proportion of megaloblasts were unlabelled in autoradiographs after incubation with tritiated leucine for 1 h. These cells remained unlabelled after long exposures of the autoradiographs so that the intensity of labelling of other megaloblasts was too high for grain counting In this paper we present the results of experiments designed to investigate the significance of these phenomena.

Methods

All radioactive compounds used for *in vitro* labelling of bone marrow were obtained from the Radiochemical Centre, Amersham, Bucks.

Gallocyanin chrome alum staining method for DNA estimation. Freshly aspirated bone marrow was labelled in vitro, in heparinised Hanks' solution containing 1.3 μCi ^{3}H-TdR/ml (specific activity = 5,000 Ci/M) at 37°C for 1 h. Marrow fragments were then smeared on glass slides, air dried and fixed in Carnoy's solution for 10 min. The smears were pre-treated with a solution containing 0.1 mg ribonuclease per ml for 2 h at 56°C, before staining. The ribonuclease (Worthington Biochemical Corporation) was made up in Sörensen's buffer (pH = 6.5). The slides were stained in gallocyanin chrome alum (pH = 1.64) for 48 h at room temperature and subsequently differentiated in running tap water for 30 min as described by SANDRITTER *et al.* [15]. The absorbance of all stained nuclei excluding those belonging to megakaryocytes, metamyelocytes and more mature white cells was measured using a Deeley pattern integrating microdensitometer at a wavelength of 5,700 Å. The positions of the measured cells were marked on a photographic map. Cells in DNA synthesis were subsequently detected by autoradiography.

Cell cycle distribution. The technique for the determination of the distribution of erythroblasts in G_1, S and G_2, using ^{3}H-TdR for the detection of DNA synthesis and the Feulgen method for the cytochemical estimation of DNA has been described previously [19]. Further experiments have been performed (1) on the bone marrows from 2 patients with vitamin B_{12} deficiency before therapy and 4 h (C.B.) and 50 h (H.K.) after a single intramuscular injection of 1 mg of cyanocobalamin and (2) using deoxycytidine 5-T (^{3}H-CdR) (2.5 μCi/ml, specific activity = 300 Ci/M) as an alternative DNA precursor. In the latter experiment portions of the same megaloblastic marrow were labelled separately with ^{14}C-TdR (5 μCi/ml specific activity = 57.9 Ci/M) and ^{3}H-CdR.

Estimation of DNA contents of cells unlabelled by radioactive amino acids. Aliquots of marrow from patients with untreated pernicious anaemia were separately labelled in heparinised Hanks' solution containing either (a) 10 μCi/ml of L-leucine-4,5-T (^{3}H-Leu) (specific activity = 1,000 mCi/mM) (b) 10 μCi/ml of L-methionine (^{3}H-Met) specific activity = 5,000 mCi/mM or (c) both ^{3}H-TdR (5 μCi/ml) and ^{3}H-Leu (10 μCi/ml). After 60 min incubation at 37°C marrow fragments were smeared on glass slides, rapidly air dried and fixed in absolute methanol for 10 min. The smears were stained by the May-Grünwald-Giemsa method and the position and type of all erythroblasts in a suitable area of the slide was recorded on a photographic map. The stain was leached out and the preparation was restained by the Feulgen method. Subsequent quantitative cytochemistry and autoradiography was performed as described previously [19]. The autoradiographs were exposed for period of 3 weeks.

Autoradiographs were also prepared of ^{3}H Leu-labelled marrow smears, prior to any staining procedure and the cells were subsequently stained through the autoradiograph using a modification of Leishman's method. These autoradiographs were used to determine the percentages of unlabelled cells in the different classes of erythroblast.

Results

The analysis of gallocyanin chrome alum (GC) stained marrows confirmed the presence of an increased proportion of G cells in the marrow in pernicious anaemia. Histograms of the GC absorbance values of erythropoietic cells not labelled with ^{3}H TdR, in 2 patients with pernicious anaemia and 2 haematologically normal persons are shown in figure 1. It can be seen that several unlabelled cells were found to have DNA contents between the 2c and 4c modes in pernicious anaemia. Such cells ('L cells') were infrequent in normal bone marrow

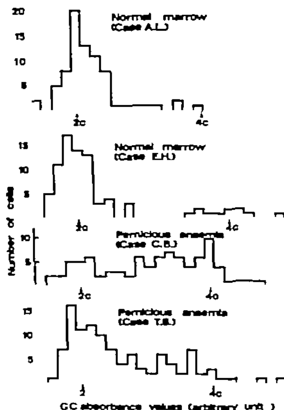


Fig. 1 The distribution of the gallocyanin chrome alum (GC) absorption values of cells not labelled with ^{3}H TdR. The cells were stained with GC after pre-treatment with ribonuclease.

Table I. The effect of using different DNA precursors for the detection of S cells in pernicious anaemia (case E.B.)

Cell type	DNA precursor	Percentages G_1	S	G_2	U	S/G_2	No of nuclei assessed
Basophilic erythropoetic cells	^{14}C-TdR	7	83	7	3	[illegible]	148
	^{3}H-CdR	14	72	10	4	7.2	150
Early polychromatic cells	^{14}C-TdR	29	38	23	10	1.7	205
	^{3}H-CdR	24	47	25	4	1.9	211

Table I shows the results of the experiment in which separate portions of the same megaloblastic marrow aspirate were labelled with ^{14}C-TdR and ^{3}H-CdR. G_1 represents post mitotic cells with a 2c content of DNA, S represents cells synthesising DNA as indicated by their incorporation of the labelled precursor and G_2 represents pre mitotic cells with a 4c DNA content after completion of DNA synthesis. Column U represents cells with DNA contents lying outside the normal spread about the 2c and 4c modes which were not labelled with ^{3}H TdR (the term U is used to indicate that the precise state of the cells is unknown) The reduced S/G_2 ratio and the presence of several U cells, in the early polychromatic megaloblasts was detected with both DNA precursors.

Sixty-two percent of basophilic erythropoietic cells in normal marrow were present in S 32% in G_1, 6% in G_2 and 0.4% in U with an S/G_2 ratio of 10.3 Normal early polychromatic cells have a higher labelling index of 81% with 11% in G_1, 8% in G_2, 0.2% in U and an S/G_2 ratio of 10.9 [19]

Table II shows the effect of a single injection of vitamin B_{12} in 2 cases of untreated vitamin B_{12} deficiency The S/G_2 ratio in the early polychromatic cells did not revert to normal in either the 24 h or

Table II. Vitamin B_{12} deficiency The effect of vitamin B_{12} therapy on the distribution of cells in interphase

Case	Cell type	Time after B_{12} h	Percentages G_1	S	G_2	U	S/G_2	No. of nuclei assessed
C.B.	Basophilic erythropoietic cells	0	10	67	17	6	3.9	157
		24	17	68	10	4	6.7	129
	Early polychromatic cells	0	15	59	16	12	3.7	127
		24	27	56	14	3	4.0	194
H.K.	Basophilic erythropoietic cells	0	13	76	5	6	15.2	126
		50	29	55	14	2	3.9	177
	Early polychromatic cells	0	17	44	23	15	1.9	81
		50	15	49	29	9	1.7	178

the 50 h marrow samples. In the basophilic cells case H K. showed a decrease in the previously normal S/G_2 ratio 50 h after commencement of vitamin B_{12} therapy In both patients there appeared to be a reduction in the proportion of cells in category U after vitamin B_{12} therapy but the difference is not statistically significant

Figure 2 shows histograms of the Feulgen absorbance values of early polychromatic megaloblasts unlabelled with radioactive amino acids in 3 patients with untreated pernicious anaemia. The relative DNA contents of unlabelled cells spread throughout the entire range between the 2c and 4c values in both the basophilic erythropoietic cells and the early polychromatic megaloblasts. Labelled cells had diploid, tetraploid and intermediate DNA values.

Figure 3 shows histograms of the relative DNA contents of unlabelled early polychromatic megaloblasts in bone marrow labelled simultaneously with ³H TdR and ³H Leu. The DNA contents of unlabelled cells (i.e. cells not synthesising either DNA or protein) were observed to be distributed both at the 2c and 4c value as well as in the entire range between the 2c and 4c modes.

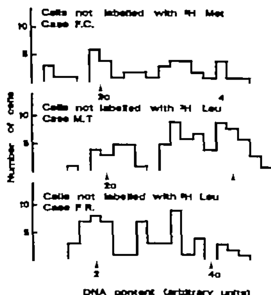


Fig 2. The distribution of DNA in the early polychromatic megaloblasts which were unlabelled with radioactive amino acids (³H Met or ³H-Leu) in patients with untreated pernicious anaemia.

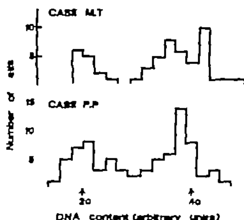


Fig 3. The distribution of DNA in the early polychromatic megaloblasts which were unlabelled with both ³H TdR and ³H Leu in 2 patients with untreated pernicious anaemia.

Table III Percentage of erythroblasts unlabelled with tritiated leucine in pernicious anaemia

Case		B.E.	E.S.	F.R.	M.T	F.C.
Haemoglobin g %		5.5	5.3	7.5	5.8	6.7
% unlabelled cells	promegaloblasts and basophilic megaloblasts	5	10	6	13	1
	early polychromatic megaloblasts	28	28	18	46	22
	late polychromatic megaloblasts	54	64	57	69	67

Table III shows the percentage of erythroblasts unlabelled with radioactive amino acids, in pernicious anaemia. The proportion of unlabelled cells was lowest in the basophilic erythropoietic cells and highest in the non-dividing late polychromatic megaloblasts. These cells remained unlabelled at a time when other megaloblasts showed intense labelling and several reticulocytes were labelled.

Discussion

So far the evidence for the abnormalities in the cell cycle in pernicious anaemia has come from the combined quantitative cytochemistry and autoradiography on individual cells. The Feulgen method has been

used for the estimation of relative DNA content and the incorporation of ^{3}H TdR *in vitro* has been used to detect cells in DNA synthesis. It may be argued, however that ^{3}H TdR is an unsatisfactory marker for DNA synthesis in pernicious anaemia as there is evidence that the tetrahydrofolic acid dependent methylation of deoxyuridylic acid to thymidylic acid is impaired in vitamin B_{12} deficiency [6 10] The use of ^{3}H TdR as a DNA label could overcome such a block and give a high labelling index so that the magnitude of the disturbances in the cell cycle would be underestimated. Evidence against the release of a block in G_1 comes from a study of the DNA content of cells labelled with H TdR. A release of a G_1 block would lead to the formation of a synchronous wave of DNA synthesis as is seen in methotrexate synchrony [12] so that after 30 min most of the cells in S would have DNA contents near the 2c mode None of the megaloblastic marrows studied showed such a distribution. The release of a block after a period in DNA synthesis would also lead to an increased labelling index with a decrease in the proportion of U cells. The results of the experiment in which both ^{14}C-TdR and ^{3}H-CdR were used in parallel to detect cells in S show that the distribution of megaloblasts in the different stages of the cell cycle was similar with either DNA precursor thus confirming the validity of the use of labelled thymidine as a marker for DNA synthesis, in pernicious anaemia. H-CdR is utilised as a DNA precursor both by its conversion to deoxycytidine triphosphate which is incorporated into DNA and via a pathway involving its deamination to deoxyuridylic acid and its subsequent methylation to form thymidylic acid [3] Unlike H TdR, ^{3}H-CdR will not circumvent the partial block in the methylation of deoxyuridylic acid to thymidylic acid in vitamin B_{12} deficiency

An increased proportion of megaloblasts in G and U was also demonstrated when the gallocyanin chrome alum method was used for the quantitative estimation of DNA. This is of particular interest because unlike the Feulgen method which depends on a partial hydrolysis of the DNA molecule and formation of aldehydes by the deoxyribose moiety the gallocyanin chrome alum complex bonds directly with the DNA phosphate groups. It would appear that the pile up of early polychromatic cells in G_2 and the presence of U cells in pernicious anaemia, is well established, being demonstrable by the use of both an alternative marker for the detection of cells in DNA synthesis and an alternative stoechiometric staining reaction for the estimation of cell DNA content.

A limitation of the consecutive cytochemical and autoradiographic technique is that it only gives information about the distribution of cells in the cell cycle at one point in time. It gives no information as to how this distribution is achieved For example, the reduction in the S/G_1 ratio in pernicious anaemia may be due to (1) a shortening of the S period (2) a prolongation of G_2, (3) a prolongation of S with a proportionally greater prolongation of G_2, or (4) an arrest of cells in G_1 leading to cell death and a combination of these mechanisms may be in operation.

Several interpretations are also possible for the U cell which has been demonstrated in pernicious anaemia but some of these can now be discarded. Feulgen microspectrophotometry cannot detect the small differences of DNA associated with the decrease of one or two chromosomes, which have been described in pernicious anaemia [7] Marked aneuploidy and bizarre chromosome numbers are not features of pernicious anaemia and the possibility that the U cell is a markedly aneuploid cell with an atypical DNA content is very unlikely Another explanation is that U cells result from a re arrangement of the histone/DNA relationship leading to an alteration in the Feulgen staining reaction. Proteins have been shown to affect the intensity of the Feulgen staining reaction and to influence the absorption spectrum of Feulgen dye *in vitro* [14] This possibility has been largely excluded by (1) demonstrating that the maximum absorbance of the Feulgen stained nuclei was at the same wave length for the U cells as for the megaloblasts in G_1, S and G_2, (2) showing that the U cell persists over a range of different hydrolysis times during the Feulgen staining procedure [16] and (3) demonstrating the presence of the U cell using the gallocyanin chrome alum method as an alternative technique for the quantitative estimation of DNA.

A further possibility is that the U cell may be explained on the basis of a model in which the commencement of DNA synthesis in individual chromosomes is staggered through the S period [1] This hypothesis was propounded to explain the finding of a significant proportion of unlabelled basophilic normoblasts in normal dogs with a continuous spectrum of DNA contents from 2c to 4c. Theoretically it would be possible for DNA replication to have been completed in some chromosomes before others commenced and for no DNA synthesis to be detected for short periods during the S period, thus explaining the above observation Against the latter mechanism is the observation that U cells are infrequent in normal human bone marrow [19]

In previous reports we suggested that the most likely explanation for the U cell is that it represents a cell which has been arrested after a period in DNA synthesis. Of the several possible explanations for the reduced S/G_2 ratio an arrest in G_2 was considered the most likely This explanation was favoured mainly in view of the strong body of evidence indicating the presence of intra-marrow cell death in pernicious anaemia, both from *in vivo* studies [8, 4 2] and *in vitro* studies [11] and also from the known role of vitamin B_{12} in DNA synthesis [15] The results of studies in protein synthesis reported in this paper give direct support in favour of this mechanism.

For the estimation of relative DNA contents of cells, in smears labelled with radioactive amino acids, autoradiographs were prepared after Feulgen staining Although a proportion of labelled protein is lost during the preliminary hydrolysis [21] Feulgen hydrolysis did not increase the proportion of megaloblasts unlabelled with tritiated leucine in pernicious anaemia The finding that cells showing very weak or no labelling after incubation with ³H TdR and ³H Leu occur at all DNA values from 2c to 4c indicates that some G cells and U cells show depressed protein synthesis. These results give added support to the view that some cells in G and U are in fact arrested in the cell cycle and are destined to intramedullary death.

A new feature which has emerged from the study of protein synthesis in megaloblasts is that the kinetic arrest in pernicious anaemia is not confined to the S and G phases of the cell cycle but biosynthetic failure occurs also in the G phase A proportion of the dividing erythroblasts in G and several non-dividing late polychromatic cells show little or no evidence of protein synthesis. These abnormalities in the G phase were not detectable by the techniques used in our earlier studies.

The results reported in this paper do not help in deciding whether the arrest in protein synthesis is the cause or a consequence of the arrest in the cell cycle A possible mechanism by which vitamin B_{12} deficiency may depress protein synthesis and lead to the arrest in the cell cycle has already been discussed [17] but this hypothesis remains to be verified.

The persistence of the cell cycle abnormalities 24 and 50 h after commencement of vitamin B_{12} therapy is consistent with the results reported by Menzies *et al* [9] and indicates that despite almost complete normoblastic conversion, several cells remain arrested in the cell cycle. These results suggest that the reticulocyte response following treatment of pernicious anaemia is largely due to the maturation of

a new generation of erythroblasts resulting from stem cell differentiation, and possibly also from the maturation of the most immature basophilic erythropoietic cells. The latter cells are least affected both as regards the arrest in G_2 and U and the arrest in protein synthesis. A similar conclusion has recently been drawn from the results of ferrokinetic studies performed on patients with pernicious anaemia during their response to vitamin B_{12} therapy [5]

Summary

The abnormalities of cell proliferation in the megaloblasts of pernicious anaemia shown in previous work have been confirmed. A proportion of megaloblasts in G_2 and the cells which were apparently arrested after a period in DNA synthesis showed little or no evidence of protein synthesis, indicating that these cells were arrested in the cell cycle and destined for an intramedullary death. A marked depression of protein synthesis was also seen in proportion of G cells. The arrest of cell proliferation in pernicious anaemia therefore occurs at all stages of interphase. The persistence of the abnormality after commencement of therapy is consistent with the idea that the reticulocyte response is largely result of the maturation of a new generation of erythroblasts or of the most immature basophilic erythroblasts which were least affected as regards both the cell cycle abnormality and the arrest in protein synthesis.

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Untersuchungen über Blut und Gewebsmakrophagen

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Makrophagen sind mononukleäre Zellen die besonders lebhaft phagozytieren und im Gewebe, in den Exsudaten seröser Höhlen und im Blut anzutreffen sind Ihre Definition gründet sich auf ihre Funktion und nicht auf ihre Herkunft. Die Frage, welche Zellarten Makrophagen ausbilden und damit zur Aufrechterhaltung der Integrität des Organismus beitragen kann noch nicht beantwortet werden [neuere zusammenfassende Darstellungen 22 37 66] Die eigenen Untersuchungen beschränken sich auf die Frage nach Herkunft und weiterer Entwicklung der Blutmakrophagen und die Beziehung dieser Zellen zu den Makrophagen bei der experimentellen Entzündung der Haut nach Rebuck [59] Beide Probleme können mit zytologischer Methode angegangen werden, die Versuchsanlage ist jedoch unterschiedlich. Im Falle der Blutmakrophagen wird mit einem definierten und geschlossenen Zellsystem gearbeitet, das eine Änderung seiner Zusammensetzung nur durch das Absterben von Zellen erfährt. Diese Versuchsanordnung ist deshalb besonders zur Beantwortung zellgenetischer Fragen geeignet Vergleichbare physiologische Verhältnisse sind begrenzt und im Blut und in Exsudaten anzutreffen Bei der Hautfenstertechnik nach Rebuck werden die Makrophagen im Gewebe beobachtet das Zellsystem ist jedoch offen und kann jederzeit durch Zu oder Abwanderung von Zellen verändert werden. Die Entscheidung zellgenetischer Probleme ist hier schwieriger die physiologische Bedeutung der Versuchsanordnung jedoch grösser da sie Aussagen über das Verhalten der Gewebsmakrophagen gestattet Beide Methoden ergänzen sich, da sie das Problem der Makrophagen unter verschiedenen Aspekten in Erscheinung treten lassen

Die Kenntnis, dass sich aus Blutleukozyten in Zellkulturen Makrophagen entwickeln, reicht bis zu den Untersuchungen von LEWIS [47] zurück. Bis heute ist jedoch noch nicht entschieden, ob diese Zellen aus Monozyten [3 36, 40 41 58 64 69] oder Lymphozyten [4 23 24 25 26 31 32, 33 34] entstehen und ob bei dieser Transformation die Anwesenheit von neutrophilen Granulozyten von Bedeutung ist [33 34] Für die eigenen Untersuchungen an Blutmakrophagen verwendeten wir menschliches Venenblut, aus dem bei spontaner Sedimentation eine Gesamtleukozytenfraktion und bei Anwendung der Trennmethode nach RABINOWITZ [58] eine Lymphozytenfraktion und eine mit Monozyten angereicherte Fraktion gewonnen wurden Damit standen verschieden zusammengesetzte und mit zytologischer sowie zytochemischer Methode definierbare Zellpopulationen als Ausgangsmaterial zur Verfügung Bei vergleichender Züchtung dieser Zellansätze wurde die Transformation zu Makrophagen morphologisch zytochemisch und quantitativ verfolgt

Methoden

Die Trennmethode nach RABINOWITZ [58] beruht auf der unterschiedlichen Haftfähigkeit der Leukozyten, die in einer mit Glasperlen gefüllten Säule inkubiert und nacheinander mit Serum und EDTA ausgespült werden. Die Lymphozytenfraktion ist nur wenig mit Monozyten und neutrophilen Granulozyten verunreinigt. Granulozyten- und Monozytenfraktionen treten kurz hintereinander aus und vermischen sich teilweise Das Trennergebnis wird deshalb um so besser je stärker die ausfliessenden Zellen fraktioniert werden. Wir verzichteten auf eine schärfere Trennung von neutrophilen Granulozyten und Monozyten, um ausreichendes Material für die verschiedenen Kulturversuche zu gewinnen. Die eigene Monozytenfraktion enthält deshalb eine starke Beimengung von Granulozyten und eine gewisse Verunreinigung durch noch nachträglich austretende Lymphozyten. Für die Züchtungsversuche standen damit folgende Zellpopulationen zur Verfügung eine Gesamtleukozytenfraktion mit einer den Blutleukozyten entsprechenden Zusammensetzung eine Lymphozytenfraktion, die ausserdem wenig Granulozyten und Monozyten enthielt und eine mit Monozyten angereicherte Fraktion, der zahlreiche Granulozyten und weniger Lymphozyten beigemischt waren. Der Prozentsatz der Monozyten aus der Relation Monozyten Lymphozyten ist für die verschiedenen Fraktionen zu Beginn von Tabelle 1 angegeben. Ein Teil der Leukozyten wird beim Trennversuch zerstört, der grösste Teil der Zellen ist jedoch morphologisch unverändert und in der Vitalität nicht merklich beeinträchtigt, d.h. Motilität, Phagozytosefähigkeit, Züchtbarkeit und Stimulierbarkeit mit Phytohämagglutinin bleiben erhalten [58, eigene Untersuchungen].

Die Züchtung erfolgte in Suspensionskultur in Reagenzgläsern unter Zusatz von Eigenserum und TC 199 mit und ohne Beigabe von Phytohämagglutinin (PHA) Zellproben wurden in ein- bis zweitägigen Abständen entnommen und auf Objektträgern ausgestrichen. Die Kulturdauer betrug bis zu 14 Tage. In parallel laufenden Kulturen wurden Deckgläser

schräg eingestellt, in verschiedenen Intervallen entnommen und die haftenden Zellen untersucht (Deckglaskulturen).

Die ausgestrichenen Zellen der Kulturansätze und der Suspensionskulturen sowie die auf den Deckgläsern haftenden Zellen wurden nach Pappenheim angefärbt. Außerdem wurden zytochemisch Peroxydase, unspezifische Esterase mit dem Substrat α-Naphthyl-Acetat [50, 51] und saure Phosphatase [29] dargestellt.

Die quantitative Auswertung der Kulturansätze und Suspensionskulturen erfolgte durch Auszählen der mononukleären Zellen untereinander, wobei in den Ansätzen Lymphozyten und Monozyten und in den Kulturproben nach 3-5tägiger Züchtungsdauer (voller Entwicklung der Makrophagen sowie Fehlen unveränderter Monozyten) Lymphozyten und Makrophagen gegenübergestellt wurden. Die schnell absterbenden neutrophilen Granulozyten blieben unberücksichtigt. Der Prozentsatz der Monozyten bzw. Makrophagen, der sich aus der Relation Lymphozyten : Monozyten und Lymphozyten : Makrophagen ergibt, ist in Tabelle I angeführt. Die quantitative Auswertung wird durch die unterschiedliche Haftfähigkeit der Zellen auf Oberflächen und ihre unterschiedliche Verteilung in Ausstrichen beeinträchtigt, erlaubt jedoch bei Anwendung von Suspensionskulturen eine bessere Aussage über das Schicksal verschieden zusammengesetzter Zellpopulationen als die einfache Abschätzung. Beim Einstellen von Deckgläsern in die Kulturen erhält man darauf eine bevorzugte Ansammlung der haftfähigen Zellen, nämlich von Monozyten, Makrophagen und stimulierten Lymphozyten. Eine quantitative Auswertung wurde deshalb hier unterlassen.

Resultate

Die Monozyten gleichen nach Passage der Trennsäule, abgesehen von einer stärkeren Neigung zur Vakuolisierung, den Verhältnissen im Blut. Nach eintägiger Züchtung sind die Zellen insgesamt etwas grösser oder breiten sich stärker aus; die Kerne sind grösser und lockerer. Im Zytoplasma, das oft wellig begrenzt ist, finden sich Phagozytose und Vakuolisierung. Nach 2 Tagen hat die Phagozytose weiter zugenommen, und einige Zellen sind völlig mit Fremdmaterial ausgefüllt, so dass der Zellkern an den Rand der Zelle gedrängt wird. Nach 3 Tagen weisen fast alle Zellen Phagozytose auf, die Zellkerne sind aufgelockert, verbreitert und vergrössert. Nach 4 Tagen ist bei einigen dieser Zellen eine weitere Transformation zu beobachten. Das Zytoplasma ist partiell oder völlig feinschaumig durchsetzt. Der Zellkern ist oval oder rund mit scharf gezeichneter Membran, liegt oft am Zellrand und lässt gelegentlich Nukleolen erkennen. Nach 5tägiger Züchtung sind die meisten Zellen wie zuvor beschrieben transformiert, phagozytiertes Material wird abgebaut, neuerliche Phagozytose ist – vermutlich mangels Materials – seltener. Die Zellen zeigen nun ein einförmiges Bild, das sie während der weiteren Züchtung bis zum Untergang beibehalten. Neben diesen Zellen treten im übrigen gleichartige Riesenzellen mit unterschiedlicher und teils hoher Kernzahl auf. Mitosen fehlen. Die beschriebene Entwicklung verläuft in Kulturen mit und ohne Zusatz der üblichen Menge von PHA gleichartig (Abb. 1 u. 2).

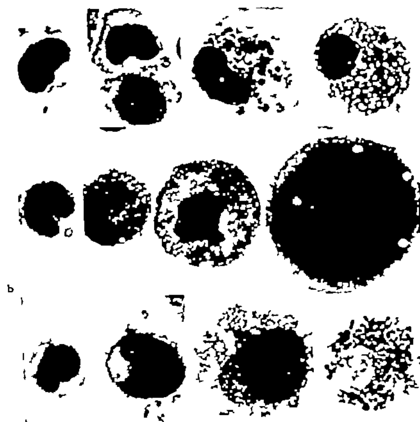


Abb. 1 Entwicklung der Monozyten in der Blutzellenkultur zu Makrophagen und Schaumzellen (× 1000). *a* Peroxydasereaktion. Zellen im Kulturansatz, nach 1-, 3- und 5tägiger Kulturdauer. *b* Unspezifische Esterase (α-Naphthylacetat, granuläres Reaktionsprodukt). Zellen im Kulturansatz, nach 3- und 5tägiger Kulturdauer, letzte Zelle zweikernig. *c* Saure Phosphatase (Reaktionsprodukt im Original rot, im Bild grau bis schwarz, Zellkerne bei 2.–4. Zelle heller als fermenthaltiges Zytoplasma). Zellen im Kulturansatz, nach 3- und 5tägiger Kulturdauer. Fermentaktivität bei Zelle 3 in Golgi-Zone. Zelle 4 beim Ausstreichen zerstört, phosphatasepositive dunkle Vesikel (Phagolysosomen) deshalb isoliert zu erkennen.

Die Lymphozyten sind nach Passage der Trennsäule unverändert. In Kulturen ohne PHA-Zusatz bewahren sie ihre Form bis zum Untergang. In Kulturen mit PHA bilden sie, beginnend mit eintägiger Kulturdauer, die bekannten morphologischen Zeichen der Stimulation mit Vergrösserung von Zelle und Zellkern, Basophilie und Vakuolisierung des Zytoplasmas sowie Kernteilungsfiguren aus. Diese Zellen phagozytieren ebensowenig wie die unstimulierten Lymphozyten.



Abb. 2. Riesenzellen (× 1000). a Mehrkernige Riesenzelle (Hautfensterpräparat). b Mehrkernige Riesenzelle und angelagerte Zellen, die ihre Zytoplasmagrenzen mehr oder weniger bewahrt haben (Riesenzellenentstehung durch Zellfusion?). Deckglaskultur. c Verschiedene Stadien von Zellkernfragmentierung (Entstehung mehrkerniger Zellen durch Amitose?). Deckglaskultur

Die Zytochemie der Zellen zeigt nach Passage der Trennsäule keine wesentliche Änderung gegenüber dem Befund, wie er von der Untersuchung im Blut bekannt ist [50, 51, 52]. Die Monozyten sind im Kulturansatz teilweise schwach peroxydasepositiv, besitzen eine deutliche Aktivität an unspezifischer Esterase sowie eine meist schwache Aktivität an saurer Phosphatase. Nach eintägiger Züchtung haben die Aktivitäten an unspezifischer Esterase und saurer Phosphatase bereits zugenommen, um in den nächsten Tagen weiter anzusteigen und mit dem 4. Tag eine maximale Reaktion zu erreichen (Abb. 1). Andere Autoren erhoben an Makrophagen verschiedener Herkunft gleichartige Befunde [3, 12, 13, 14, 15, 16, 17, 18, 19, 24, 25, 32, 69]. Mit einsetzender Phagozytose, die im gegebenen Milieu vorwiegend absterbende neutrophile Granulozyten betrifft, finden sich dicke, tropfige Einschlüsse peroxydasepositiven Materials, das in der Folge wieder abgebaut wird, so dass nach dem 5. Tag, wenn die meisten neutrophilen Granulozyten abgestorben und bereits phagozytiert sind, auch die Peroxydaseaktivität nachläßt und in immer weniger Zellen nach

Tabelle 1. Prozentuale Anteile der Monozyten bzw. Makrophagen aus der Relation Monozyten : Lymphozyten (Kulturansatz) bzw. Makrophagen : Lymphozyten (nach 3–5-tägiger Kulturdauer)

Lymphozytenfraktion	ohne Zusatz von PHA					mit Zusatz von PHA				
% Monozyten im Kulturansatz	<1	3	3	4	5	<1	3	3	4	5
% Makrophagen nach 3–5 Tagen Kultur	1	<1	<1	5	2	<1	<1	1	3	1
Granulozytenfraktion	ohne Zusatz von PHA					mit Zusatz von PHA				
% Monozyten im Kulturansatz	17	12	18	18	17	17	12	18	18	17
% Makrophagen nach 3–5 Tagen Kultur	10	10	12	16	16	<1	<1	1	10	4
Monozytenfraktion	ohne Zusatz von PHA					mit Zusatz von PHA				
% Monozyten im Kulturansatz	82	72	73	50	93	82	12	73	50	93
% Makrophagen nach 3–5 Tagen Kultur	89	58	70	54	86	90	4	85	16	97

mißglückter Trennversuch

gewiesen werden kann. Die zytochemischen Befunde sind in Tabelle II dargestellt, sie zeigen in Kulturen mit und ohne PHA Zusatz keine signifikanten Unterschiede

Die Lymphozyten enthalten keine Peroxydase keine oder nur vereinzelte Granula von unspezifischer Esterase und keine oder nur selten saure Phosphatase die dann fleckförmig lokalisiert ist In Kulturen ohne PHA Zusatz bleiben die Lymphozyten auch bei einer Versuchsdauer von über 14 Tagen zytochemisch unverändert. Bei Zusatz von PHA zeigt ein Teil der deutlich stimulierten Zellen keine Änderung der Fermentaktivität *Andere stimulierte Zellen weisen eine* Zunahme der Aktivitäten von unspezifischer Esterase und saurer Phosphatase auf, die jedoch deutlich hinter der Aktivität der Monozyten gleicher Züchtungsdauer zurückbleibt (Tab II)

Das Ergebnis der quantitativen Auswertung ist in Tabelle I angegeben. Der Anteil der Makrophagen ist in der Lymphozytenfraktion niedrig in der Gesamtleukozytenfraktion etwas höher und in der Monozytenfraktion am höchsten wobei eine Abhängigkeit von den in den Kulturansätzen vorliegenden Monozytenzahlen besteht. Wird durch PHA Zugabe eine stärkere Proliferation der Lymphozyten angeregt, dann sinkt der Anteil der Makrophagen entsprechend ab Die Relation Monozyten Makrophagen wird durch die Zahl der neutrophilen Granulozyten die in der Gesamtleukozytenfraktion sowie der Monozytenfraktion hoch, in der Lymphozytenfraktion dagegen niedrig ist, nicht erkennbar beeinflusst.

Bringt man in eine Suspensionskultur zugleich oder nach einer gewissen Latenzzeit Deckgläser in schräger Stellung ein (Deckglaskultur) dann sammeln sich darauf besonders die haftfähigen Zellen nämlich Monozyten Makrophagen und stimulierte Lymphozyten an während neutrophile Granulozyten und nicht stimulierte Lymphozyten deutlich zurücktreten Auch auf den Deckgläsern bilden sich zunächst Makrophagen aus die morphologisch und zytochemisch den Makrophagen der Suspensionskulturen gleichen. Ebenso sind bei Fortsetzung der Züchtung die zuvor beschriebenen einförmigen Zellen mit rundem oder ovalem Kern und feinschaumigem Zytoplasma zu beobachten Daneben ist jedoch eine zunehmende Neigung der Makrophagen zu amöboider Beweglichkeit festzustellen mit Ausbildung breiter Pseudopodien durch welche die Zellen in die Länge gezogen werden Die sich begegnenden Zellen haften aneinander und ziehen bei weiterer Fortbewegung die verbindenden Zytoplasmabezirke zu langen fadenförmigen Strängen aus, so dass eine Ansammlung

Tabelle II Aktivitäten unspezifischer Esterase und saurer Phosphatase bei Lymphozyten und Monozyten im Kulturansatz und in Kultur mit und ohne Zusatz von Phytohämagglutinin (PHA)

Kulturdauer	Lymphozyten ohne PHA unspezif. Esterase	saure Phosphatase	mit PHA unspezif. Esterase	saure Phosphatase	Monozyten ohne PHA unspezif. Esterase	saure Phosphatase	mit PHA unspez. f. Esterase	saure Phosphatase
Kulturansatz	0–(+)	0–(+)	0–(+)	0–(+)	+	(+)	+	(+)
1 Tag	0–(+)	0–(+)	0–+	0–(+)	+ ++	+	+ ++	+
2 Tage	0–(+)	0–(+)	0–+	0–(+)	+ ++	+ +	+–++	++
3 Tage	0–(+)	0–(+)	0 +	0 +	++	++ +++	++	++ +++
4 Tage und länger	0–(+)	0–(+)	0 +	0 +	+++	+++	+++	+++

Bei der Hautfenstertechnik nach Rebuck ist eine morphologische und zytochemische Ähnlichkeit der mononukleären Zellen mit den Blutmonozyten unverkennbar, während deutliche Unterschiede gegenüber den Lymphozyten bestehen. Weitere Hinweise auf die Genese der Zellen liefert die histologische Untersuchung des Entzündungsareals. Bereits von Bürgener [11] beobachtete eine Ansammlung monozytenähnlicher Zellen im Gefässlumen und ihre Auswanderung in das Entzündungsgebiet. Leder und Crespin [43] konnten diesen Befund bei histologischer Untersuchung von Exzisionsmaterial bestätigen, das am Ort der nach Rebuck angelegten Hautfensterentzündung gewonnen wurde. Dagegen waren primär im Gewebe nur wenig Zellen nachzuweisen, die sich bei Darstellung der unspezifischen Esterase wie Monozyten verhielten. Diese Befunde sprechen ebenso wie Markierungsversuche mit Isotopen [6, 27, 65, 68] dafür, dass die mononukleären Zellen bei der experimentellen Entzündung der Haut vorwiegend hämatogener Herkunft sind und – bei Berücksichtigung der zytomorphologischen und zytochemischen Kriterien – Monozyten darstellen. Mit der Transformation dieser Zellen zu Makrophagen bildet sich eine völlige morphologische und zytochemische Übereinstimmung mit den Makrophagen der Blutzellenkultur aus, wobei sich auch der zeitliche Ablauf etwa gleichartig vollzieht.

Die Aktivität an saurer Phosphatase, zunächst ein Leitsymptom der Makrophagenentstehung, ist neben anderen sauren Hydrolasen ein Kennzeichen der 1955 von de Duve [21] beschriebenen Lysosomen, denen eine besondere Bedeutung bei der intrazellulären Degradation biologischen Materials zukommt. Es wird angenommen, dass die Lysosomen in der Golgizone entstehen, in das Zytoplasma übergehen und sich dort unter Zunahme ihres Volumens mit phagozytiertem oder pinozytiertem Material zu Phagolysosomen vereinigen [neuere Übersicht 63]. Elektronenoptisch konnte in Makrophagen eine Zunahme der Lysosomen an Zahl und Grösse vor und während der Phagozytose festgestellt werden [15, 16, 64]. Die eigenen Untersuchungen lassen bei Darstellung der sauren Phosphatase ebenfalls einen Orts- und Gestaltwechsel der Lysosomen erkennen. Einige Makrophagen enthalten die Fermentaktivität isoliert in der Golgizone, andere Zellen besitzen eine im Zytoplasma diffus verteilte Reaktion. Werden Makrophagen beim Ausstreichen zerstört, dann zerfällt das Zytoplasma in Venkel verschiedener und auch besonderer Grösse, die sich durch ihre starke Fermentaktivität als Lysosomen bzw. Phagolysosomen zu erkennen geben (Abb. 1c).

Die mehrkernigen Riesenzellen in Suspensionskulturen, auf Deckglaskulturen und im Hautfensterpräparat gleichen sich völlig Sie gehen aufgrund ihrer morphologischen und zytochemischen Charakteristika *offensichtlich aus Makrophagen bzw Monozyten hervor* Mitosen sind nicht zu erkennen, ihre Genese kann sich deshalb durch Zellfusion oder durch Amitose vollziehen. Unsere Untersuchungen erlauben zunächst keine Entscheidung da sowohl verschiedene Stadien von Zellzusammenlagerung und Verschmelzung als auch Kernfragmentationen zu beobachten sind. In Suspensionskulturen und auf Deckgläsern bilden die Makrophagen oft Zellkonglomerate in denen Zellgrenzen nur noch stellenweise erkannt werden können (Abb 2) Auch bei elektronenmikroskopischer Untersuchung ist eine Auflösung der Zellgrenzen festzustellen [64] Diese Befunde sprechen für Riesenzellbildung durch Zellfusion [28 30 47 64 69] Andererseits sind sowohl bei der Hautfenstermethode als auch besonders auf Deckglaskulturen *verschiedene Stadien von Zellkernfragmentation bis zur völligen* Teilung zu erkennen, ohne dass die Zellen nekrobiotische Veränderungen erleiden (Abb. 2) Diese Beobachtungen können als Hinweis auf den Ablauf von Amitosen angesehen werden, wobei eine DNS-Vermehrung vor oder nach der Fragmentation eintreten kann [35] Wichtig sind die Untersuchungen von GOLDSTEIN [30] der in monozytären Riesenzellen Einzelkerne mit hypo- aber auch mit hyperdiploidem DNS-Gehalt fand. Die amitotische Entstehung von Riesenzellen des Hautfensters wird insbesondere von LEDER [42, 46] vertreten [allgemeine Ausführungen zur Genese von Riesenzellen 49]

Unabhängig von der gewählten Methode wandeln sich die Monozyten in der Suspensionskultur auf Deckgläsern und im Hautfenster versuch zunächst zu Makrophagen und Riesenzellen um, wobei morphologische und zytochemische Identität besteht. Bei Fortsetzung der Untersuchungen sind jedoch weitergehende Zelltransformationen zu beobachten, die unterschiedlich ablaufen und mit einer gewissen Gesetzmässigkeit zu einer zusätzlichen Typisierung führen. Da das zelluläre Ausgangsmaterial gleichermassen aus Monozyten besteht, müssen diese Differenzierungsvorgänge durch die unterschiedliche Versuchstechnik bedingt sein. In der Suspensionskultur treten vom 4 Züchtungstag an die oben beschriebenen Zellen mit breitem, schaumigem Zytoplasma und häufig randständigem ovalem Zellkern auf, die etwas ungenau als Epitheloidzellen bezeichnet werden, treffender aber u. E. als Schaumzellen charakterisiert werden können. Züchtet man die gleichen Zellen auf Deckgläsern, d h. auf einer Unter

lage, dann entwickeln sich teilweise ebenfalls Schaumzellen andere Zellen lassen jedoch eine zunehmende amöboide Fortbewegung erkennen und nehmen den Status von Wanderzellen an. Im Hautfensterpräparat schliesslich wandeln sich die Makrophagen zu bindegewebigen Elementen um, die sich wie Epitheloidzellen, mit denen sie zytochemisch übereinstimmen [42] oder Fibroblasten zusammenlagern. Diese experimentell provozierten Transformationsformen und Funktionszustände besitzen ihre Entsprechungen *in vivo* So konnten wir in Pleura und Peritonealexsudaten neben monozytären Makrophagen [67] ebenfalls Schaumzellen auffinden, die morphologisch und zytochemisch den Befunden in Blutzellkulturen gleichen. Bei der lokalen Entzündung nehmen die Monozyten den Funktionsstatus der Wanderzellen an, dringen in das Entzündungsgebiet ein und können es wiederum als Makrophagen verlassen, um in die regionären Lymphknoten zu gelangen [22] Die bindegewebige Transformation von Makrophagen ist in ihrem gesamten Ablauf bei histologischer Untersuchung des Entzündungsgebietes zu beobachten [22, 61]

Phagozytose ist eine verbreitete Zelleigenschaft und nicht auf Monozyten beschränkt. Die gut überschaubaren Versuchsmodelle der Blutzellzüchtung und der Hautfensterentzündung zeigen in Analogie zu entsprechenden Beobachtungen *in vivo* dass sich unter den besonders zur Phagozytose befähigten Zellen ein einheitliches System befindet, das im Blut, in den Exsudaten seröser Höhlen und im Gewebe Makrophagen auszubilden vermag Diese Zellen werden im Blut als Monozyten und im Gewebe als Histiozyten [1 39] bezeichnet. Sie schliessen sich zu einem genetisch einheitlichen System der Histiomonozyten [7–10] zusammen. Die ganze Breite ihrer Funktion ist bei der lokalen Entzündung zu erkennen, wo sie als Wanderzellen, Makrophagen und Riesenzellen am Ort der Schädigung auftreten, gemeinsam mit den neutrophilen Granulozyten der Abwehr dienen und nach Abräumen des Entzündungsfeldes zusammen mit dem lokalen Bindegewebe die Reparation einleiten.

Zusammenfassung

Vergleichende Untersuchungen mit der Blutzellenkultur und der artefiziellen Hautentzündung nach Rebuck zeigen, dass die hierbei zu beobachtenden Makrophagen aus Monozyten entstehen. Unter den verschiedenen experimentellen Bedingungen bilden die Monozyten bzw Makrophagen weitere Transformationsformen aus, die ihre Entsprechung in Differenzierungsvorgängen *in vivo* finden.

Summary

Comparative studies of blood cell cultures and artificial inflammation of the skin according to Rebuck have shown that the macrophages seen in these circumstances develop from monocytes. Under various experimental conditions, the monocytes or macrophages go through further stages of transformation which have counterparts in vivo in processes of differentiation.

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Loss of Acetylcholinesterase Activity in Human Erythrocytes Treated with Cephalothin[1]

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In an earlier communication [1] we reported that erythrocyte acetyl cholinesterase (ACHE) activity was subnormal in newborn infants affected with ABO hemolytic disease, but not in those affected with Rh disease. This finding has been confirmed by several workers [2–4]. The activity of this stromal enzyme is also reduced in patients with paroxysmal nocturnal hemoglobinuria (PNH) [5] However studies with red cells separated in accordance with their density revealed that in this disorder the enzyme distribution pattern was characteristically different than that found in ABO hemolytic disease [6] Investigations undertaken to elucidate the pathophysiology of this enzyme defect have shown that alterations of ACHE activity can be produced by exposing normal erythrocytes to certain chemically-unrelated substances which interact with the red cell membrane [7–9] From inactivation studies with proteolytic enzymes it has been inferred that ACHE appears to be located at the outer surface of the human erythrocyte [5]

We have recently shown that *in vitro* treatment of normal red cells with benzylpenicillin resulted in irreversible inactivation of ACHE [10] This effect was demonstrable with concentrations of antibiotic which are commonly used in sensitizing erythrocytes for the detection of anti-penicillin antibodies. The reports of immunohematological cross-reactivity between penicillin and the semi-synthetic antibiotics derived from cephalosporin C [11–16] as well as the suggestion of red

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cell membrane alterations [17] caused by cephalothin [sodium salt of 7 (thiophene-2-acetamido)cephalosporanic acid] prompted an investigation of the effects of this substance on erythrocyte ACHE. For comparison, red cells were also exposed to two analogues of cephalothin cephaloridine [7 (2-thienyl acetamido) 3-(1-pyridylmethyl) 3-cephem-4-carboxylic acid betaine] and cephaloglycin [7 (D-α-aminophenylacetamide) cephalosporanic acid zwitterion] Glucose-6-phosphate dehydrogenase (G6PD) was used as a marker to assess an effect on intracellularly located enzymes [7] While this work was in progress, SIRCHIA *et al.* [18] reported that cephalothin at a concentration of 40 mg/ml converted normal erythrocytes into PNH-like cells i.e. the cells had become susceptible to lysis in acidified normal serum (positive Ham test) *However these investigators did not examine the* action of the antibiotic on ACHE. In this communication we present evidence that the treatment of normal human red cells with cephalothin is associated with an irreversible loss of ACHE activity

Materials and Methods

Blood obtained from normal adult individuals was centrifuged at 1,500 rpm for 5 min and the plasma and buffy coat were removed by suction. The erythrocytes were washed 3 times with 20 volumes of ice-cold 0.1 M sodium-potassium phosphate buffer pH 7.0. After the last centrifugation 50 percent cell suspension in the same buffer was prepared. Separation of red cells into young and aged populations was carried out as previously described [6]. When the influence of isoantibodies was investigated, washed blood-group A, Rh erythrocytes were agglutinated with excess of anti-A or anti-D (Ortho Diagnostics); dispersed, washed again 3 times and adjusted to 50 percent suspension. Hemoglobin-free cell membranes were prepared by osmotically-induced hemolysis and stored at 4 C as 50 percent suspension.

Antibiotic stock solutions were prepared daily in phosphate buffer Unless otherwise indicated, 5 volumes of correspondingly diluted antibiotic solution were added to one volume of 50 percent erythrocyte suspension and incubated at 37°C. Cells treated with buffer alone served as controls. At the end of the incubation period, erythrocytes were washed thrice with up to 100 volumes of chilled buffer and 50 percent suspension was prepared after the last centrifugation. ACHE activity was measured at 412 nm on replicate 0.1 percent cell suspensions in 0.1 M phosphate buffer pH 8.0 using acetylthiocholine as substrate and 5 5 -dithiobis-(2-nitrobenzoic acid) as color reagent [1]. Specific activity was expressed as ΔOD/min/mg hemoglobin. G6PD activity was determined according to the method of ZINKHAM [19]. Hemoglobin was measured at 540 nm as cyanmethemoglobin.

Results

Incubation of intact erythrocytes with cephalothin at 37°C and pH 7 0 caused a concentration-dependent loss of ACHE activity

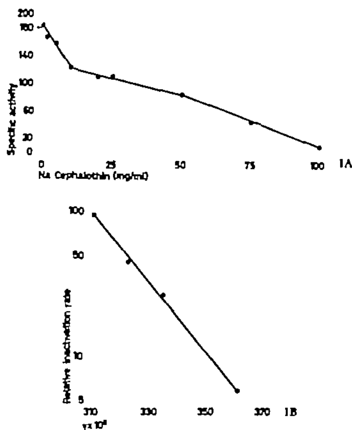


Fig 1 Effect of concentration and temperature on ACHE inactivation by cephalothin. *A* To replicate 0.2 ml of 50 percent cell suspension in 0.1 M phosphate buffer pH 7.0, 1.0 ml of cephalothin solution in the same buffer was added. Following incubation at 37°C for *60 min, cells were washed 5 times and ACHE activity was measured as indicated in the text.* *B* At 4°C, 1.0 ml of cephalothin (50 mg/ml) was added to replicate 0.2 ml of 50 percent cell suspension. After incubating for 60 min at the temperatures indicated, the cells were washed thrice and adjusted to 50 percent suspension. Residual enzyme activity was related to controls incubated without antibiotic.

(fig 1A) Whereas the effect was moderate at low concentrations, approximately 50 percent of the enzyme activity was destroyed by 50 mg/ml of cephalothin. Essentially similar results were obtained when incubation was carried out at pH 6.0 or pH 8.0. Repeated washing of *cephalothin-treated erythrocytes did not restore* ACHE activity and none was detected in the supernatant liquid following incubation with cephalothin. Hemolysis was only noted with the highest concentrations of the antibiotic. However red cell integrity

was not necessary for the inactivating effect of cephalothin, because the enzyme of cell-free membrane preparations behaved like that of intact erythrocytes. No differences in ACHE inactivation were seen with erythrocytes from individuals of different blood-groups and with cells segregated by density into young and aged populations. The effect of the antibiotic appeared to be restricted to the red cell membrane, since the activity of G6PD an intracellularly located enzyme, was not affected by incubating intact erythrocytes for 180 min at 37°C and pH 7.0 with 50 mg/ml of cephalothin.

As can be seen in table I, when whole blood was incubated with cephalothin, only minimal reduction in ACHE activity was noted. Similarly treating washed erythrocytes with the antibiotic in the presence of blood-group antisera did not cause a significant loss of enzyme activity. However when blood-group A, Rh^+ red cells were agglutinated with anti-A or with anti-D washed free of excess antibody and then exposed to cephalothin, the loss in ACHE activity was as great as that seen with non-agglutinated erythrocytes (table I)

Additional information on the interaction of red cell ACHE and cephalothin was obtained by studying other parameters which influence enzyme inactivation. Thus, the effect of temperature was determined between 4 C and 45 C and the relative inactivation rates are shown in figure 1B. At the concentration used, 94 percent of the enzyme activity was destroyed at 45 C, while at 4 C only a 5 percent loss was noted. When erythrocytes were treated with cephalothin

Table I. Effect of cephalothin on ACHE of blood-group A, Rh positive erythrocytes

Conditions of erythrocytes	ACHE activity	
	Cephalothin	Control
Whole blood	108	138
Washed	22	142
Agglutinated with anti-A	24	142
Agglutinated with anti-D	23	142

Heparinized blood from an adult individual was divided into 2 aliquots. One aliquot was stored at 4°C and the erythrocytes of the other aliquot were washed and suspended in buffer Washed cells were agglutinated with an excess of antisera, dispersed, washed again 3 times and adjusted to 50 percent suspension. Whole blood and cells were incubated at 37°C for 180 min with 5 volumes of cephalothin (50 mg/ml) After 3 additional washings with phosphate buffer ACHE activity was measured at pH 8.0.

solutions which had been heated for 120 min at 37° C, inactivation of ACHE was not affected. The action of cephalothin on the enzyme was time-dependent. In figure 2A it can be seen that whereas 50 per cent of the activity was destroyed in 60 min, 21 percent remained after 180 min at 37° C. The relatively slow loss of ACHE activity suggested

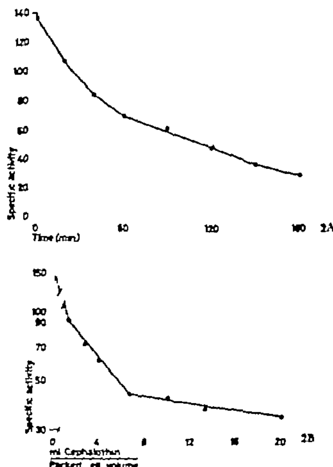


Fig. 2. Effect of time and erythrocyte concentration on ACHE inactivation by cephalothin. *A* At 4° C, to replicate 0.2 ml of 50 percent cell suspension, 1.0 ml of cephalothin (50 mg/ml) was added. After incubation at 37° C for time indicated, the cells were washed 3 times and stored as 50 percent suspension at 4° C. ACHE activity was measured simultaneously as indicated in the text. *B* To replicate tubes containing increasing amounts of 50 percent erythrocyte suspension (range 0.1 ml to 1.5 ml) 1.0 ml of cephalothin (50 mg/ml) was added. Antibiotic was omitted in controls. Following incubation at 37° C for 120 min, cells were washed thrice and ACHE activity was determined as indicated. Abscissa computed with packed cell volume.

that inactivation was also dependent on the concentration of erythrocytes. This possibility was therefore tested and it was found that when the concentration and volume of cephalothin were kept constant, the reduction in ACHE activity was inversely proportional to the amount of cells present (fig 2B)

In order to obtain some information about the specificity of the action of cephalothin on ACHE, red cells were also exposed to two of its microbiologically active analogues. Incubating intact erythrocytes for 180 min at 37° C and pH 7 0 with up to 100 mg/ml of cephaloridine, the pyridine analogue, did not result in reduction of ACHE activity The relative insolubility of the phenyl glycine analogue, cephaloglycine, prevented a more systematic study however at a concentration of 5 mg/ml this substance was equally ineffective.

Discussion

The foregoing experimental results have demonstrated that the exposure of human erythrocytes to cephalothin resulted in inactivation of the cell membrane ACHE. The failure to restore enzyme activity by repeated washing of the antibiotic-treated cells indicated that the effect was irreversible, thereby resembling the inactivating action observed with benzylpenicillin [10] The present findings are also reminiscent of the effects of proteases [7] and of tannic acid [20] on erythrocyte enzymes that is, only the membrane associated ACHE, but not the intracellularly located G6PD was destroyed when whole red cells were exposed to the antibiotic. These observations suggest that cephalothin only interacts with specific segments of the outer surface of the human erythrocyte. As previously seen with tannic acid [9] the action of cephalothin on ACHE was also neutralized by the presence of proteins in the incubation mixture. However enzyme activity was not protected by agglutinating erythrocytes with blood-group specific antibodies prior to their exposure to the antibiotic.

MOLTHAN *et al.* [17] have shown that the addition of cephalothin to washed erythrocytes produced a positive antiglobulin reaction. The strength of this reaction was related to the length of incubation and to the concentration of antibiotic used. In the present study we found that ACHE inactivation was not only dependent on time and concentration, but also on the red cell concentration and on the temperature of incubation. SIRCHIA *et al.* [18] have suggested that the

positive Ham test evoked by cephalothin could be due to modifications of membrane proteins which resemble the alterations occurring spontaneously in the PNH erythrocyte. Our results complement and extend their findings by demonstrating that ACHE activity which is characteristically reduced in PNH [5] was destroyed when red cells from normal individuals were exposed to cephalothin. Although the mechanism by which this substance exerts its action on ACHE has not been elucidated, the conversion of normal erythrocytes into enzyme deficient cells could lead to a better understanding of PNH and of ABO hemolytic disease.

Acknowledgement

The antibiotics were generously provided by Dr. L. D. Bechtol of Eli Lilly and Co.

Summary

Treatment of human erythrocytes with cephalothin caused irreversible inactivation of the surface located acetylcholinesterase (ACHE). The effect on the enzyme was dependent on concentration, time and temperature. ACHE inactivation was prevented by plasma but not by agglutinating the red cells with blood-group specific antibodies. The intracellularly located glucose-6-phosphate dehydrogenase was not affected by cephalothin. Cephaloridine and cephaloglycin had no effect on ACHE.

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Note Added in Proof

Since this paper was submitted for publication, Ferrone *et al.* (Europ. J Pharmacol. *4* 211 [1968]) have reported that the exposure of whole blood to cephalothin caused reduction in erythrocyte ACHE activity

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Plasma Clearance of Heated Serum Bound Co^{57} Vitamin B_{12} [1]

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It has been previously shown that there is a specific pattern of plasma clearance of intravenously administered tracer doses of radioactive B_{12} in normal persons, patients with pernicious anemia (PA) in relapse, untreated chronic myeloid leukemia (CML), myeloid metaplasia (MM) and fish tapeworm anemia (FTA) [1, 2, 3, 4, 5, 6]. Injections of similar amounts of labeled vitamin B_{12} prebound to normal serum have no effect on the clearance rate in normal subjects or patients with CML and MM whereas in untreated PA and FTA, the slow clearance is converted to normal [5, 6]. It was suggested that normal serum contains a 'clearance factor' absent in some patients with PA and FTA in relapse and that this 'factor' is regenerated after intensive therapy with parenteral large doses of vitamin B_{12} [5, 6].

Materials and Methods

In the present study, Co^{57}-vitamin B_{12} (specific activity greater than 10.0 µc/µg) was added to normal serum in a ratio of 1.0 ng/ml, and incubated at room temperature for 1 h. An aliquot was removed and tested with charcoal to demonstrate complete binding [7]. Thereafter, the entire sample of serum and Co^{57}-B_{12} was placed in a 56°C water bath for 30 min. At the end of this period, another aliquot was removed and treated with charcoal to demonstrate complete B_{12} binding, following which the contents were rapidly infused i.v. Samples of blood were obtained from subjects at 1, 2, 5, 15, 30, 45 and 60 min and 2, 4, 8, 12, 24 and 48 h. Retained plasma activity was calculated on the basis of venous hematocrit, height and weight of each donor, as previously described [5, 6].

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Table I. Percent of injected radioactivity in plasma of normal persons (N) and patients with pernicious anemia (PA) and fish tapeworm anemia (FTA) receiving free and normal serum (NS) prebound with $Co^{57}B_{12}$

		1 h, %			24 h, %			48 h, %		
Normal (range)		11.12 (7.2–14.0)			4.9 (3.5–7.0)			3.4 (2.2–4.8)		
Name	Diagnosis	$Co^{57}B_{12}$	$Co^{57}B_{12}$ + NS	$Co^{57}B_{12}$ + NS (heated)	$Co^{57}B_{12}$	$Co^{57}B_{12}$ + NS	$Co^{57}B_{12}$ + NS (heated)	$Co^{57}B_{12}$	$Co^{57}B_{12}$ + NS	$Co^{57}B_{12}$ + NS (heated)
AA	N	11.3		20.5	4.2		8.8	3.2		7.0
HE	N	10.2		19.1	3.2		7.9	2.4		6.3
KI	N	10.2		18.9	3.1		6.8	3.0		3.3
DO	FTA	19.8	10.7	16.9	10.7	3.8	9.2	8.9	2.7	8.5
HA	FTA	21.6	11.3	16.0	12.1	3.4	6.9	10.1	3.2	3.2
KO	FTA	23.6	10.3	25.6	14.1	3.7	9.8	11.3	2.4	5.2
TA	FTA	18.6	8.6	17.0	12.0	3.1	9.6	7.3	1.9	7.0
SU	PA	21.0	13.2	22.3	12.2	4.7	10.2	9.3	2.9	7.7
HK	PA	14.7		20.4	8.8		8.9	5.9		5.5
LE	PA	19.0		18.5	10.3		8.6	8.5		6.4
MA	PA	13.6		24.9	4.0		10.6	2.7		7.6

partially treated

Results

The data are presented in table I. In normal persons, the vitamin prebound to the individual's own serum and heated to 56°C for 30 min, induced a clearance rate in the slow range. Similarly, when Co^{57}-B_{12} was prebound to normal serum, subjected to heat and administered to patients with PA (treated and untreated), the clearance rate was delayed. There is ample experience from our own laboratory thus far that administered normal serum bound with Co^{57} B_{12} will consistently change a slow rate to normal in patients with PA and FTA in relapse [5, 6]. In all instances of FTA and a single case of PA, the slow clearance rate of Co^{57} B_{12} was converted to normal when labeled vitamin was prebound to normal serum. When the same serum and vitamin were heated to 56°C for 30 min and injected i.v., the clearance pattern reverted to the slow rate.

Discussion

The data suggest that there may be, in reality, a specific vitamin B_{12} clearance factor which is heat labile. However, there would then be adequate clearance factor in the normal recipients and the PA patients with normal clearance rates to replace the heated factor and maintain the clearance rate in the normal range. What is more likely is that the serum B_{12} protein complex is so altered by heat that the vitamin is not in an available form and the ensuing clearance resembles that of CML and MM, both of which show slow patterns uncorrectable by normal serum of the donor [5].

Summary

Normal serum, prebound with Co^{57}-vitamin B_{12} heated to 56°C for 30 min and injected intravenously into normal subjects and patients with pernicious anemia in relapse, is followed by a slow disappearance rate over a period of 24 h when compared to the unheated complex. The possible implications of these observations are discussed.

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Adverse Effects of Steroids in Acute Myeloblastic and Monoblastic Leukemia[1]

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It has been generally accepted that steroids have their best therapeutic effect in acute lymphoblastic leukemias [4, 8]. In acute granulocytic leukemia, the steroids have fared consistently poorly in inducing remissions or prolonging survival. Steroids, may in fact, accelerate the course of acute granulocytic leukemia [1–3, 5–7, 9–17]. This has been reported to occur less frequently when steroids are combined with one or more myelosuppressive agents [11].

This study reviews the therapeutic treatments of acute myeloblastic and monoblastic leukemia with and without steroids. Observation of poor treatment effects and high incidence of acceleration of disease in patients treated with steroids alone as well as in conjunction with a myelosuppressive agent, prompted this report.

Materials and Methods

The records of all patients with acute myeloblastic, monoblastic or myelomonoblastic leukemia admitted to Queen's Hospital between July 1957 to December, 1967 were studied. Patients with chronic granulocytic leukemia in blast crisis were not included.

Thirty-one patients with acute myeloblastic and monoblastic leukemia were included in this study. Twenty-four received steroids alone or in combination with a myelosuppressive agent and 4 were treated with 6-mercaptopurine alone. Three others died without any treatment. The response to therapy was evaluated by criteria established by Clinical Studies Panel, Cancer Chemotherapy National Service Center [7]. The term 'improvement' denoted diminution in WBC count (and abnormal cells), increase in hemoglobin and/or

[1] This study was supported by a grant from research funds of Queen's Medical Center, Honolulu.

platelets which were insufficient in extent or duration to classify that as partial remission. I certain cases, no abnormal immature cells were noticed on peripheral smear and hemoglobin, platelet count had also improved but there was no bone marrow examination available at this time to be certain about completeness or remission. Such cases have been included in category of partial remission.

Treatment

A. Steroid group. The term 'steroid' treated group included patients in whom steroids alone or in conjunction with myelosuppressive agent were used in an attempt to induce remission. *In one patient, however, steroids were given* initially with mistaken diagnosis *of drug rash* The steroids used included hydrocortisone, prednisone, prednisolone, triamcinolone decadron and ACTH. The myelosuppressive agent used was 6-mercaptopurine except in 2 cases where methotrexate was used. The dose of steroids used was usually the equivalent of 40–60 mg prednisone daily and it ranged from 15 to 100 mg per day

B. Non-steroid group. Patients in whom a myelosuppressive agent alone was used to induce remission. In some cases, steroids were used later during relapse in small doses for bleeding tendencies. *The myelosuppressive agent used was 6-mercaptopurine in doses of* 2.5–3.0 mg/kg/day

Aggravation of leukemia was characterized by sharp increase in leukocyte count and deterioration in clinical condition, with one or more of features like increase in bone pains, bleeding tendency anemia and thrombocytopenia immediately following institution of steroids. Many of these patients died rapidly I some cases, the leukocyte count was controlled by 6-mercaptopurine, and this appeared to have delayed death. However in others, death ensued despite control of the WBC count by 6-mercaptopurine

Results

Two patients in steroid group (8.3 %) and one patient in non-steroid (8.3%) were less than 20 years 9 patients in steroid group (37.5 %) and 6 patients in non-steroid group (50 %) were between 20 and 50 years and the remaining 13 patients (54 1 %) and 5 cases (41 6 %) in each group respectively were over 50 years of age at the time of diagnosis. Among the patients in whom platelet count was available at the time of diagnosis 17 out of 18 in steroid group and 8 out of 11 in non-steroid group had platelet count less than 100 000/mm 62.5 patients in the steroid group and 66.6% in the non-steroid group had less than 10,000 blasts per mm^3 33.5% and 25.0% patients in first and second group respectively had 10 000–100,000 blasts per mm^3 while the remaining 4 1% and 8.3 % respectively in 2 groups had more than 100,000 per mm^3

Three patients (12.5%) in the steroid group and one patient (8.3 %) in the non-steroid group had significant bleeding Among the 3 cases in the former group, 2 had vaginal bleeding and one had subdural hematoma at the time of diagnosis in the latter group one patient (8.3%) had marked epistaxis at the time of diagnosis. Thus, the

Table I. Comparison of response to treatment of patients in steroid and non-steroid groups

Treatment	Number of patients	Complete remission	Partial remission	Some improvement	No response	Aggravation of leukemia
Steroids alone	4	0	1 (child)	0	1	2
Steroids and single myelosuppressive agent	20	0	1	2	8	9
Myelosuppressive agent alone	12	0	5	3	4	0

Chi-square analysis comparing the non-steroid group (myelosuppressive agent alone) and steroid group (steroids alone or with myelosuppressive agent) revealed the difference in remission rate to be significant statistically ($P<0.05$)

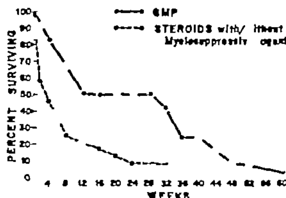


Fig 1

patients in the 2 groups were comparable as regards the various factors affecting ultimate survival.

Survival period. Comparison of ultimate survival in the 2 groups revealed striking differences The median survival from onset of first symptoms to death was 8 0 weeks in the steroid treated group and 16.0 weeks in non-steroid group The difference appears still more pronounced when the comparison is made between the median survival period from the time of diagnosis (fig 1) This period was 2.8 weeks in the *steroid group and 13 weeks in the non-steroid group.* The ultimate survival of central $^2/_3$ patients in the 2 groups also revealed longer

survival time in patients treated with 6-mercaptopurine alone. Eighty four % patients in non-steroid group died within 60 weeks, whereas the same proportion of patients in steroid group were dead by 20 weeks from onset.

Remissions. Table I shows the response to treatment with different therapeutic regimens. There was no complete remission in the patients presently studied Partial remission was achieved in only 2 of the 24 patients receiving steroids. One of these 2 patients was an 11 year old child treated with steroids alone. Compared to steroid group patients treated with 6-mercaptopurine (non-steroid group) enjoyed more frequent remission. Five among 12 patients had partial remission. In some of these the response was fairly close to complete remission. At the time of best response, bone marrow examination revealed blast cells constituting 6 % of total nucleated cells.

Acceleration of leukemia. Eleven out of 24 patients (46%) receiving steroids, had aggravation of the course of disease soon after the start of treatment. Six among the 11 cases died within 18 days of starting therapy (range 3 days – 18 days) Two patients received corticosteroids alone without myelosuppressive agents. The cause of death in these was intracerebral and bilateral adrenal hemorrhage respectively In another patient 6-mercaptopurine was started but patient died before any appreciable effect of this drug was noticed. The other 3 patients died even when the leukocyte count was declining One of these had jaundice at the time of death and thus was considered to be related to the hepatotoxic effect of 6-mercaptopurine Two other patients had severe endometrial hemorrhage and intracerebral hemorrhage respectively

In the remaining 5 patients the acceleration of the disease was controlled to a varying extent by the effect of 6-mercaptopurine and they died 4.5 12.0 6 0, 21.3 and 5.5 weeks respectively after treatment. The favorable effect of 6-mercaptopurine in this group was seen only after the 5th day of administration. In certain cases, it was delayed up to the 9th–11th day after starting treatment.

It was notable that continuation of steroids, after the 6-mercaptopurine effect had been obtained, did not alter the further drop in leukocyte count. However if the dose of steroids was now increased suddenly the leukocyte count increased in 3 cases (fig 2–3)

The dose of steroids used was usually equivalent to 40–60 mg of prednisone. However in some cases even as little as 15 mg (fig 2) and 20 mg (fig 3) was enough to cause acceleration of disease.

While no complete remissions were observed, partial remission was seen in 41 6% of the patients treated with 6-mercaptopurine alone and in only 8.3% of the cases in the steroid treated group The myelosuppressive agents, however appeared to suppress the deleterious effects of steroids in the present study The suppressive action of 6-mercaptopurine was apparent, only after the 5th and sometimes only after the 9th–11th day of therapy coinciding with the onset of action of 6-mercaptopurine The aggravating effect of steroids appeared more rapidly occasionally within a few hours, in several cases in the present series

Summary

Results of treatment of cut myeloblastic and monoblastic leukemia with therapeutic regimens containing steroids and with 6-mercaptopurine alone have been compared in retrospective study Among 24 patients in the steroid treated group, there were partial remissions in only 2 cases, the median survival time was 8 weeks from first symptoms (2.8 weeks from diagnosis) and there were 11 cases (46%) in which distinct evidence of acceleration of disease was noted. Compared to these, 12 patients in non-steroid group (treated with 6-mercaptopurine) had 5 partial remissions and their median survival time was 16 weeks from onset of first symptoms (13 weeks from diagnosis) The use of steroids appeared to have accelerated the course of acute myeloblastic and monoblastic leukemia.

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Two Families with Different Expression of Homozygous β-Thalassaemia

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It is now believed that thalassaemia represents a group of haemoglobinopathies characterized by partial or complete depression of synthesis of one of the polypeptide chains. Since 5 types of polypeptide chains are normally synthesized by man (α, β, γ, δ, ε) 5 types of thalassaemia could theoretically exist. Two of them, α- and β-thalassaemia are now well documented. Delta and gamma-thalassaemia have recently been described [7, 8, 9]. Epsilon-thalassaemia has not yet been found. Recently Schroeder *et al.* [13] presented evidence for the existence of more than one structural gene for the γ-chains.

Several different types of β-thalassaemia are now recognized with the differentiation being based on haemoglobin F and A_2 values: (1) with elevated Hb A_2 or A_2 thalassaemia; (2) with normal level of Hb A_2 but increased Hb F or F thalassaemia (β/δ-thalassaemia) and (3) with normal levels of Hb F and Hb A_2 [6, 11, 15]. The homozygous state for A_2-thalassaemia is usually associated with 20 to 60% of Hb F, while occasionally levels of less than 10% and more than 90% of Hb F have been found [1]. These findings have been seen in 40 carriers of β-thalassaemia (A_2-thalassaemia) treated in the Department of Pediatrics, Medical School of Skopje, since 1952 [12]. The present report describes the clinical and haematologic findings in 4 patients with A_2-thalassaemia, 2 of which had a moderately to very severe form of the disease with biochemical findings different from those usually found in A_2-thalassaemia (marked elevation of Hb A_2 and a small increase of Hb F) and present evidence for further heterogeneity of β-thalassaemia.

Methods

Standard laboratory procedures were used for total haemoglobin concentration, packed cell volume, red cell counts, reticulocyte counts, blood smears stained with Wright's stain, and osmotic fragility [16]. For the demonstration of fetal haemoglobin inside red blood cells the technique developed by Betke and Kleihauer [2] was used. Starch gel electrophoresis wa carried out in the usual way [14] in tris-EDTA-boric acid buffer pH 8.9 [5]. Quantitation of Hb A_2 was performed by starch block electrophoresis according to the technique of Kunkel and Wallenius [10] in tris-EDTA(disodium salt)-boric acid buffer pH 9.1 [4]. The alkali resistant haemoglobin was determined by the method of Betke *et al.* [3].

Case Reports

Case 1 (fig. 1 and table I, III-2) This girl was admitted to the Department of Pediatrics, Medical School of Skopje, for the first time in 1964 at the age of 9 years. Before that time she had been treated on several occasions in local hospital for anaemia, subicterus, weakness, fatigue, frequent respiratory infections anorexy and bdominal pain. Physical examination at the time of admission revealed severe anaemia, dark colored skin, icterus, pronounced mongoloid facies, and retardation in growth. The liver was enlarged up to umbilicus and the spleen down to symphysis. Radiographic examination showed pronounced cardiomegaly X-ray of the skull revealed typical picture of 'tete en brosse' and decalcification of the long bones. Haematological data Hb 4.9 g%; RBC $2.25 \times 10^6/mm^3$; haematocrit 18%; reticulocytes 12% increased osmotic fragility (0.48–0.26% NaCl) marked aniso- and poikilocytosis, numerous target cells, anisochromia and nucleated red cells. Determination of the red blood cells survival with ^{51}Cr revealed half-life of 8 days. No

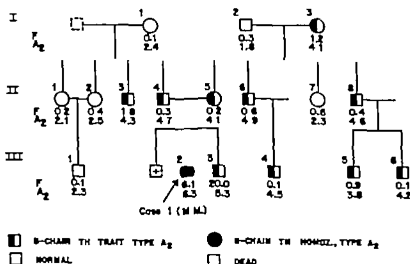


Fig. 1 Pedigree of family 1 case 1 (III-2).

G-6-PD deficiency was observed. The distribution of fetal haemoglobin inside the red cells was unequal. On the basis of these findings and because of the constant need for transfusions, the patient was diagnosed as a Cooley's anaemia (homozygous classical thalassaemia) of severe degree.

Table I. Haematologic data on family 1 case 1 (III-2)

Case No.	Age years	Hb g%	RBC ×10⁶/mm³	Hct. %	MCH pg	Hb F %	Hb A_2 %
I-1	72	14.1	4.[illegible]60	43	30	0.1	2.4
I-2	66	15.1	4.790	45	31	0.5	1.8
I-3	61	13.9	4.850	44	28	1.2	4.1
II-1	29	12.1	3.770	33	31	0.2	[illegible]
II	25	13.8	4.300	40	[illegible]	0.4	2.5
II-3	31	[illegible]	4.320	[illegible]	78	1.8	4.3
II-4	49	[illegible]	4.260	36	78	0.3	4.7
II-5	33	14.4	4.860	44	29	0.2	4.1
II-6	31	13.1	4.970	41	27	0.6	4.9
II-7	35	14.8	4.870	40	30	0.6	[illegible]
II-8	49	13.6	4.620	41	29	0.4	4.6
III-1	7	12.8	4.260	40	30	0.1	[illegible]
III-	9	4.9	2.[illegible]0	18	1	6.1	6.3
III-3	6	10.1	–	37	–	70.0	5.5
III-4	13	11.4	4.590	39	76	0.1	4.5
III-5	9	[illegible]	4.590	40	25	0.9	3.8
III-6	21	13.5	4.610	[illegible]	27	0.1	[illegible]

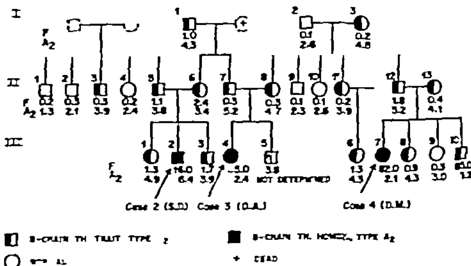


Fig. 2. Pedigree of family 2 case (III-2), case 3 (III-4), case 4 (III 7).

Starch gel electrophoresis revealed Hb A. Hb A_2 was 6.3% and Hb F 6.1%. Similar investigations made 3 years later revealed 7.8% of Hb A_2 (determined by DEAE-Sephadex chromatography) and 4.7% Hb F (determined by the method of Betke et al. [3]).

Three generations including 17 relatives were studied; the family data and pedigree are shown in table I and figure 1 The 6 year old brother (III 3) presented clinical findings of thalassaemia intermedia. He was slightly anaemic and subicteric with suggestively mongoloid facies. The liver was just palpable and the spleen was enlarged by 2 cm below the costal margin. Hb F was 20% and Hb A_2 5.3%. The boy never required blood transfusion.

Case 2 (fig. 2 and table II III-2) The propositus was 7 year old boy Up to 4 years of age no visible signs except mild pallor had been noted. He was treated on several occasions at the local hospital because of weakness, fatigue headache bdominal pain, anorexia periodic fever and mild subicterus. On physical examination he was found to have mild pallor and subicterus. Liver and spleen were enlarged by 1 and 2 cm, respectively below the costal margin. The skull and long bones ppeared normal on X-ray Laboratory data indicated moderate anaemia and moderate changes in red cell morphology Hb 8.1 g%; RBC 3.2×10^6/mm³ haematocrit 21%; reticulocytes 4% increased osmotic fragility (0 44–0.28% NaCl); Hb A_2 6.4% and Hb F 16%.

Table II. Haematologic data on family 2 case 2 (III-2) case 3 (III-4) case 4 (III 7)

Case No.	Age years	Hb g%	RBC $\times 10^6$/mm³	Hct. %	MCH pg	Osm. resist. % NaCl	Hb F %	Hb A_2 %
I 1	63	12.9	4.780	41	27	0.50–0.30	1.0	4.3
I-2	60	13.6	4.600	45	29	0.48–0.34	0.1	2.6
I-3	60	10.8	4.120	40	26	0.46–0.28	0.2	4.8
II 1	53	16.8	5.400	55	31	0.44–0.34	0.2	1.3
II-2	48	14.0	4.640	50	30	0.44–0.34	0.8	2.1
II-3	38	14.7	5.000	42	29	0.44–0.28	0.3	3.9
II-4	36	13.3	4 480	38	29	0.48–0.34	0.2	2.4
II-5	42	12.9	4.380	42	28	0.44–0.30	1 1	3.8
II-6	38	13.5	4.720	41	28	0.40–0.30	2.4	3.4
II 7	33	11.0	4.300	38	25	0.50–0.30	0.3	5.2
II-8	30	14.0	4.820	45	28	0.52–0.30	0.3	4.7
II-9	16	11.2	3.780	40	30	0.48–0.34	0.1	2.3
II 10	13	12.2	3.990	40	30	0.48–0.34	0.1	2.6
II 11	28	10.1	3.960	39	25	0.50–0.28	0.2	3.9
II 12	36	11.8	4.200	35	28	–0.28	1.8	5.2
II-13	35	10.1	3.860	38	26	0.44–0.28	0.4	4.1
III 1	8	11.2	4.180	38	26	0.42–0.32	1.3	4.9
III 2	7	8.1	3.200	21	25	0.44–0.28	16.0	6.4
III 3	4	13.8	4.800	40	28	0.40–0.32	1.7	3.9
III-4	5	6.7	2.850	–	23	–0.26	55.0	2.4
III-5	1	–	–			–	3.8	–
III-6	4	10.1	3.660	36	27	0.50–0.30	1.3	4.3
III 7	4	1.8	0.850	–	21	–	82.0	2.1
III-8	3	10.0	3.820	36	26	0.42–0.28	0.9	4.3
III-9	2	10.1	3.580	36	29	0.50–0.34	0.3	3.0
III 10	2.5m	7 7	2.900	25	26	–	85.0	1.2

Case 3 (fig. 2 and table II, III-4): This patient is a cousin of case 2. She was hospitalized for the first time in 1967 at age of 3 years, because of growth retardation mild anaemia, jaundice and abdominal pain. On physical examination she presented pronounced pallor and subicterus. The facies was mongoloid. The liver was enlarged by 2 cm and the spleen by 3 cm below the costal margin. The heart was slightly enlarged with an apical systolic murmur. Haematological data: Hb 6.7 g%; RBC $2.85 \times 10^6/mm^3$; reticulocytes 6.4%; anisocytosis, anisochromia, poikilocytosis, hypochromia. Haemoglobin electrophoresis revealed the presence of Hb A, Hb F and Hb A_2. Because of these observations and of the close relationship with case 2, additional family members were studied (fig. 2; tab. II).

Case 4 (fig. 2 and table II, III 7): This patient was admitted to the Department of Pediatrics at the age of 3 years; she is the second cousin of case 2. She had frequent respiratory infections since early childhood. She showed a pronounced pallor subicterus, lassitude, periodic abdominal pain, headache, an enlargement of the liver and spleen up to the umbilicus, pronounced mongoloid facies, and retardation in the growth (height 86 cm, weight 11.6 kg). Radiological examination showed generalized osteoporosis and beginning of 'tête en brosse'. Haematological data: Hb 1.8 g%; RBC $0.85 \times 10^6/mm^3$ marked anisocytosis, anisochromia, poikilocytosis and hypochromia; Hb F 82% and Hb A_2 2.1%. On the basis of the clinical and haematologic data, this patient was diagnosed as Cooley' anaemia of severe degree.

Discussion

This investigation was performed on 2 families. In the first family 16 members of 3 generations were studied. In 10 of them microcytosis, mild poikilocytosis, hypochromia and an increased osmotic fragility of the red cells were found. Hb A_2 was increased in all 10 members, while Hb F was slightly increased in only 3 members. An exception was case III 3 a brother of the propositus (case 1) who showed a marked elevation of Hb F (20%) with 5.3% Hb A_2. Clinical and haematological findings suggested a thalassaemia intermedia.

The second family consisted of 3 smaller but closely related families, each having a member with homozygous thalassaemia.

Twenty two members were studied 16 showed morphological changes of the red cells with an increased osmotic fragility and increased levels of Hb A_2. Hb F was increased in 9 members. The distribution of Hb F over the red blood cells was unequal in all cases studied.

Based on the severity of the disease, the 4 cases described in detail seem to fall into 2 groups A severe form of thalassaemia major (cases 1 and 4) and a milder form of thalassaemia major (cases 2 and 3) The biochemical findings also differed in the 4 patients. In cases 1 and 2 low levels of Hb F (6.1 and 16.0%) with an increase of Hb A_2 (6.3 and 6.4%) were found, while in cases 3 and 4 the levels of Hb F were 55 and 82% and those of Hb A_2 2.4 and 2.1%. These

similarities in the biochemical findings do not correspond with the clinical observations. It seems therefore that the genetic constitution of these patients is even more heterogeneous than originally suspected. All 4 can be considered as homozygotes for β-thalassaemia (A_2-thalassaemia) but because of the low quantity of Hb F in case 1 with an increased level of Hb A_2, and the severity of the disease, another genetic form of thalassaemia cannot be excluded. The severity of the disease in this patient could be explained by the presence of a gene for an abnormal haemoglobin in addition to a gene for β-thalassaemia. Such an abnormal haemoglobin, if present, should have the same electrophoretic and chromatographic properties as Hb A or Hb A_2. Appropriate laboratory tests ruled out the presence of the following haematological disorders hereditary spherocytosis, autoimmune haemolytic anaemia, paroxysmal haemoglobinuria, iron deficiency anaemia and deficiency of G-6-PD

The biochemical findings in case 2 were similar to those in case 1 except that the level of Hb F was considerably higher (16 %) On the other side the degree of the disease was milder Cases 3 and 4 can also be considered homozygotes for classical β-thalassaemia the severity of their disease, however was different although they were relatives.

A severe anaemia in patients with an apparent classical β-thalassaemia can also be explained by different types of β-thalassaemia genes, resulting in a different rate of production of the β-chains. However the significant difference between 2 members of the same family both with an apparent homozygous β-thalassaemia, makes it almost necessary to assume that other genetic modifiers may be present but to our knowledge this is impossible to prove.

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Summary

Clinical and biochemical observations made on 4 patients with homozygous β-thalassaemia are presented. Studies of 2 families, one with 17 members and the second consisting of 3 smaller closely related families, each having case of homozygous β-thalassaemia (A_2-thalassaemia) are also reported. In two of the homozygotes high levels of Hb A_2 (6.3 and

6.4%) with relatively low levels of Hb F (6.1 and 16%) were observed, while the severity of the disease differed. In the other two patients the severity of disease was also different. The clinical, haematological and biochemical observations made it likely that different forms of β-thalassaemia were present in these patients.

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Haemoglobin D in a Greek Family[1]

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Haemoglobin D is characterized by its electrophoretic mobility which on paper or on starch block electrophoresis at pH 8.6 is identical with that of haemoglobin S. Hb D on agar gel electrophoresis at pH 6.5 has the same mobility as haemoglobin A. Moreover Hb D can be distinguished from Hb S as in the reduced state in phosphate buffer it is much more soluble than haemoglobin S and the erythrocytes which contain it cannot be induced to sickle.

Haemoglobin D was described by ITANO in 1951 in a family of North American origin. A number of Hb D cases has been reported since then in people of various origin as American Negroes, Indians, Persians, Turks etc. Haemoglobin D is found with considerable frequency in North West Indians and particularly in Sikhs Punjabi Hindus and Gujeratis [7]

The majority of the reported haemoglobin D cases are either simple heterozygous, that is Hb D trait, or double heterozygous cases of Hb D in combination with the gene of sickle cell anaemia, or with the gene of thalassaemia, whereas homozygous cases are very few

In Greece a case of Hb D trait has been reported by GOUTTAS *et al.* [8] Therefore this is the first study of all members of a Greek family with haemoglobin D

Methods

Sickling tests were done by the method of DALAND and CASTLE. Foetal haemoglobin was estimated by the method of alkali denaturation for 1 min. The solubility test was done as described by ITANO. Electrophoresis of haemoglobin was carried out on 2 starch blocks 22 x 9

[1] Paper presented before Greek Haematological Association at Thessaloniki on December 1966.

cm with barbital buffer on current 250 V for 14 h. Blood cell counts, haemoglobin estimation and other routine laboratory studies were done in the usual manner

Case Reports

Propositus. We first investigated the youngest member of the family (K. K.) who entered the clinic on 4. 12. 65 for diarrhoeic syndrome He is quite a tall and strong young man of 22 years, who on clinical examination showed only a slight pallor on his face and enlargement of his spleen three fingers below the costal margin.

The blood film showed numerous target cells, a slight anisocytosis and poikilocytosis and few microcytes. No normoblasts were found. The skull X-ray was normal. On starch block electrophoresis at pH 8.6 the haemoglobin migrated as single band like haemoglobin S whereas the sickling test was invariably negative The solubility of this haemoglobin in phosphate buffer at 25 C was in the normal rate After that a diagnosis of D haemoglobinosis was entertained and we extended the investigation to the remaining members of the family the father the mother an older brother and sister Two female and the last male children of the family died before reaching the age of 1 year

Family members. The findings of the haemoglobin electrophoresis are as follows (fig 1) 1 The haemoglobin of the *father* (Pr K.) consists of single band of Hb D. The haemoglobin of the *mother* (O. K.) looks normal, consisting of Hb A and 3% Hb A_2. 3. The haemoglobin of the elder *brother* (P K.) separates into 2 fractions, 58% migrating in position A and 42% in position D. This person therefore, presents Hb D trait. 4 The haemoglobin of the *sister* (M. K.) consists of single band of Hb D. The laboratory data of the members of family K are summarized in table I

The physical condition of all the members of the family is good, and except for the father who is relatively short, the other members of family K are tall and strong. The propositus, the father and the sister reveal considerable enlargement of the spleen 3 fingers below the costal margin. The mother has moderate enlargement of the spleen about 2 fingers below the costal margin whereas no enlargement of the spleen has been found on the elder brother

The family originates from a village on the Pindos mountains of an altitude of 2000 m above sea level. After completing our study we sent a blood sample of the propositus to the

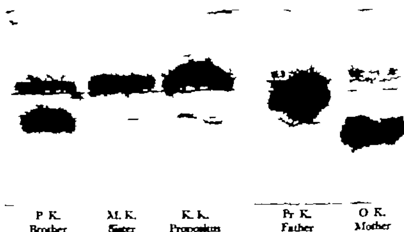


Fig 1 Starch block electrophoresis of haemoglobins from the family K.

Table I Laboratory data of the members of family K.

	Age years	RBC 10^6/mm^3	Hb g%	PCV %	MCV μ^3	MC-HC %	Osmotic fragility % NaCl	Retic. %	Target cells	Hb F %	Bilirubin mg%	Electrophoretic pattern	Sickling	ABO	Rh
Pr. K., father	59	5.65	12.6	45	80	28	0.40–0.20	1.3	many	1.7	1.0	D	neg.	O	
O. K., mother	60	4.85	11.4	42	86	27	0.40–0.28	1.5	few	2.3	0.9	A	neg.	O	+
P. K., brother	29	5.35	15.7	53	99	30	0.44–0.32	0.8	non	1.5	0.5	A+D	neg.	O	+
M. K., sister	25	5.85	12.2	45	76	27	0.40–0.20	2.3	many	3.8	1.0	D	neg.	O	+
K. K., propositus	22	6.50	13.4	49	75	27	0.40–0.20	1.6	many	2.9	1.3	D	neg.	O	+

Abnormal Haemoglobin Research Center in Cambridge where the identity of the haemoglobin was confirmed. *The fingerprint showed that this sample was haemoglobin D Punjab.* We wish to thank Dr. H. Lehmann, director of the above center for his kindness and help.

Discussion

The propositus K. K., before the family study was completed could be considered as a case of homozygous haemoglobin D disease, as in the electrophoresis on starch block his haemoglobin moved in a single band to the position S/D whereas the sickling test repeatedly was negative. When mixed with normal haemoglobin this haemoglobin in starch block electrophoresis separated into two fractions, one in position A and the second in position S/D. Furthermore, this haemoglobin was completely soluble in 2.24 M phosphate buffer whereas the haemoglobin of sickle cell anaemia was insoluble in the same buffer forming the well known precipitate.

As the brother looked like Hb D trait while the sister gave the same findings as the propositus, we could suppose that the propositus and his sister were homozygotes and the brother heterozygote for Hb D. This hypothesis can be excluded because the father giving a single band in the S/D position, looks like homozygote, whereas in the mother's blood no Hb D could be detected. Another explanation for these findings is the combination of the haemoglobin D gene with another abnormal gene, especially that of β-thalassaemia, which can simulate the homozygous condition of haemoglobin D.

It has been pointed out that the gene of thalassaemia when associated with another gene of certain haemoglobinopathies inhibits the production of the normal haemoglobin A. In these haemoglobinopathies the haemoglobin is an abnormal β-chain variant of haemoglobin A, and the depression of the production of haemoglobin A is due to the fact that β-thalassaemia only depresses the formation of normal β-chains. The interaction between β-thalassaemia and an abnormal β-chain haemoglobin is due to the fact that β-thalassaemia specifically interferes only with haemoglobins containing the normal β-chain [9]. This phenomenon, for example, is observed in sickle-cell thalassaemia where because of the interaction of the genes of Hb S and β-thalassaemia, the production of haemoglobin A is depressed and the haemoglobin of these persons consists almost exclusively of haemoglobin S and F. Thus, in the family here described, the propositus (K. K.) and his sister (M. K.) constitute 2 cases of double hetero-

zygosity that is a combination of β-thalassaemia and Hb D whereas the brother (P. K.) is a case of Hb D trait. As far as the mother is concerned, the anisocytosis and poikilocytosis, the few target cells and the basophilic stippling of her erythrocytes could establish the diagnosis of β-thalassaemia minor.

The father's haemoglobin on the electrophoresis gives a single band in the position D. According to the above data the father could be Hb D homozygote or double heterozygote of Hb D and β-thalassaemia. This could not be decided, because a study of his family was not possible. We have only an indication about the genotype from the other data. Thus the parents (Pr. K. and O. K.) have had 3 other children which died below the age of one year. The parents say that they had leukemia but it is difficult to accept this diagnosis made without proper control in a small town 30 years ago. Most probably they died from a homozygous condition of congenital haemolytic anaemia, obviously from homozygous β-thalassaemia, having inherited a gene from each parent.

Summary

A Greek family with Hb D-thalassaemia is described. Two children present a combination of Hb D and β-thalassaemia genes and the third child is a Hb D trait. The mother has β-thalassaemia minor; the genotype of the father is uncertain.

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G. Tyteca: **Cytogenèse des mégacaryocytes extramédullaires.** Arscia, Bruxelles 1968. 131 p.

Der Autor hat es sich zum Ziel gesetzt, die Entstehung extramedullärer Megakaryocyten zu untersuchen. Der erste Teil umfasst eine ausführliche Literaturübersicht, die sich auf die Beobachtungen beim Tier wie beim Menschen erstreckt. Im zweiten Teil werden nach einer kurzen Erläuterung der vorwiegend histologischen Techniken die eigenen Tierversuche näher beschrieben. Daraus geht hervor, dass lokale Gewebsnekrose oder allgemeine Hämolyse bzw. die Freisetzung von Hämoglobinabbauprodukten wesentliche Voraussetzungen für die Neubildung von Megakaryocyten in der Leber der Maus darstellen. Ein kurzes Kapitel ist zellkinetischen bzw. autoradiographischen Studien gewidmet. Die Untersuchungen beim Menschen nehmen nur einen kleinen Platz ein. Bei 6 Fällen wurden die Megakaryocyten in verschiedenen Lungenabschnitten gezählt. Dabei ergaben sich ausserordentlich grosse Unterschiede ohne Zusammenhang mit dem Grundleiden. Die Histologie vermag keinen Hinweis auf die Rolle der pulmonalen Megakaryocyten im Plättchenhaushalt und auf ihren eventuellen Einfluss auf die pulmonale Zirkulation zu geben; nur dynamische Methoden könnten näheren Einblick in diese Beziehungen gewähren. Während die embolische Verschleppung der pulmonalen Megakaryocyten aus dem Knochenmark nicht bezweifelt wird, nimmt der Autor für die Entwicklung von Megakaryocyten in andern Organen (Leber, Milz usw.) eine Entstehung *in loco* aus Retikulumzellen an. Als Hauptargumente für diese Annahme führt er zytologische und topographische Kriterien an. Es steht noch nicht fest, ob eine solche extramedulläre Neogenese von Megakaryocyten auch beim Menschen vorkommt.

Die Monographie vermittelt eine Fülle von Daten, welche sorgfältig ausgewertet und mit vorzüglichen Mikrophotographien dokumentiert worden sind. Da es sich um ein sehr *spezielles Teilgebiet der Zytogenese handelt,* werden die Ausführungen vor allem den experimentell tätigen Hämatologen oder Pathologen interessieren.

U. Bucher, *Bern*

K. S. Ranganathan: **Essentials of Blood Grouping and Clinical Applications.** Grune & Stratton, New York 1968. 178 p. Price: US-$ 6.00.

The book is written to help blood bank technicians in the routine examinations before and after transfusions and in the antenatal and postnatal immunological studies. The author, consultant of the Blood Bank of the Medical Centre of Madras, uses the RH-Hr nomenclature of Alexander Wiener (who wrote the foreword).

G. Rosenow, *New York, N.Y.*

Ch. M. Zmijewski (with the assistance of J. L. Fletcher and R. L. S. Pierce): **Immunohematology.** Appleton-Century-Crofts, New York 1968. 300 p.

This book on immunohematology provides the type of information that was urgently needed by teachers and students of this complicated field alike. The author likes to think of immunohematology as the study of immunology in relation to the elements of the blood, rather than the study of pathogenic phenomena involving blood group immunologic mechanisms. Thus, blood group systems as well as leucocyte antigens and antibodies were treated as an example of immune reactions. Accordingly, chapter 2, following the history of blood transfusion, summarizes current concepts of antigens, antibodies and immune reactions. The following chapters give an up-to-date account on the ABO blood group system, secretion of blood groups, the MNS and P blood group systems, the Rh blood groups, the application of the antiglobulin test, I blood groups as well as genetic aspects of immunohematology. The appendix describes step-by-step procedures of most widely used immunologic methods, including leucocyte grouping. Tables summarizing phenotypic frequencies of human blood groups as well as a careful subject index conclude the volume. This book on immunohematology constitutes an example of how a modern text-book can and should be written.

E. Beck, *Basel*

Acta haemat. 41: 129–134 (1969)

Departamento de Medicina A and Departamento de Medicina B, Hospital Universitario and Instituto Venezolano de Investigaciones Científicas, Caracas

Mobilisation of Iron by Desferrioxamine in Cases with Low Serum Iron[1]

J. J. Desenne and M. Layrisse

Since the isolation of desferrioxamine B (DF) from *Streptomyces pilosus* by Bickel *et al.* in 1960 [1] this substance has been widely used for the removal of excessive iron from the body stores [2–4]. Because of the remarkable properties of DF to bind iron from the deposits and its rapid excretion by the urine, several authors [4–11] have used this compound to diagnose pathological deposits of iron or iron depletion in the body. We determined the excretion of iron in patients with low serum iron either due to iron loss or other causes and in apparently normal subjects.

Material and Methods

Fifty eight subjects divided into 4 groups were studied.

Group A: 8 hematologically normal males with minor problems were chosen from the medical and orthopedic wards. Group B: 18 hematologically normal females from the medical and orthopedic wards, the out-patient department and among volunteers from the Medical School. Group C: 21 patients with iron deficiency anemia due to hookworm infection, prolonged gastrointestinal or vaginal bleeding. Some had received blood transfusions before the test, however, none had been treated with iron. Group D: 11 patients with different types of carcinomas, pulmonary tuberculosis or chronic pulmonary inflammatory diseases with anemia and serum iron below 70 µg/100 ml.

Routine blood morphology was performed according to standard techniques. Plasma iron was determined according to 2 methods [12, 13] and the total iron binding capacity (TIBC) was measured as such [14] or as unsaturated iron binding capacity [15]. Bone marrow aspiration was performed in all the subjects except 8 medical students from group B, and the smears were stained for iron [16].

The urine iron was determined by a slight modification of the method of [illegible] and

[1] Supported in part by 'El Fondo de Ayuda Económica del Departamento de Medicina A, Hospital Universitario', Caracas, Venezuela.

Fafar [17]. As the readings were usually low 10 to 40 ml of urine were used instead of the 5 ml of the original technique.

A slow intravenous injection of 500 mg of DF[a] dissolved in 10 ml of sterile water was given to all the patients. Subsequently the urine was collected in wide neck bottles rendered iron free and containing 1 ml of HCL. Hemoglobin in the urine was negative in all samples.

Results (fig 1)

Group A The average 12 hour excretion of iron in the males following the injection of 500 mg of DF was 609 $\mu g \pm 110$ This figure was not significantly different from the one found by Biederman *et al.* [7] (P >0 1) Group B The females had an average 12 hour excretion of 373 $\mu g \pm 115$ the difference of excretion between apparently normal males and females was significant (P< 0 01) Among those in whom bone marrow examination was performed 4 had no stainable iron in their

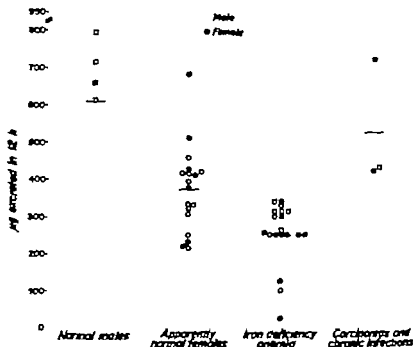


Fig. 1 Twelve hour urinary iron excretion after desferrioxamine in the different groups of subjects.

[a] As Desferal® generously supplied by Dr J Ruttelaert Ciba, Basle, Switzerland

bone marrow Only one had a transferrin saturation below 20 which might suggest an iron deficiency Three of these 4 women had the lowest excretion of the whole group. Group C The 21 patients with iron deficiency anemia had a mean serum iron of 45 μg/100 ml and a mean TIBC of 396 μg/100 ml the percentage of transferrin saturation was below 17 in all cases except one (19%) None had stainable iron in the bone marrow The average excretion of iron in the 12 hour urine was 250 μg $\pm$ 81 This value is significantly smaller than the one obtained in normal males ($P < 0.01$) but not statistically different from the value obtained in apparently normal females ($P > 0.2$) Group D All the subjects of this group had a low serum iron which averaged 43 μg/100 ml, most TIBC were diminished except for one patient, who had a high value. The mean index of transferrin saturation was 19%. The stainable iron in the bone marrow ranged from traces to +++ The 12 hour urinary iron averaged 525 μg $\pm$ 189 Comparison of these subjects with iron deficient patients revealed a significant difference ($P < 0.01$) Only 2 patients had a low excretion, one of them had only traces of iron in her bone marrow

Comments

Between the normal males and apparently normal females there was a significant difference in the excretion of iron after the injection of DF Eight of 18 females had a urinary iron within the range of iron deficiency anemia patients. These findings are similar to those reported by others [10 18] FIELDING *et al.* [18] by estimating the amount of ferrioxamine chelated *in vivo* found that 35% of apparently normal females had values within the limits for patients with iron deficiency anemia. WÖHLER [10] in a 6 hour urinary excretion test found a lower excretion in males (0.398 mg) than in females (0.264 mg) BIEDERMAN *et al.* [7] however did not find a significant difference. In our group B, the 4 women who had no hemosiderin in the bone marrow with a low iron excretion and no anemia would fit into the category which DAGG *et al.* [11] term sideropenic.

The presence of a low serum iron and anemia usually suggests the diagnosis of iron deficiency anemia. Even the association of hypochromic anemia, low serum iron, low percentage of transferrin saturation and elevated iron binding capacity is occasionally found in

anemias due to inflammation or tumors [19] In our patients of group D the low percentage of transferrin saturation and the low serum iron could have suggested an iron deficiency anemia, but in all of them except one the TIBC was low The higher iron excretion after DF established the difference between this group and the iron deficiency anemia subjects. In the patients with chronic infection studied by Wöhler [10] the excretion of iron was somewhat higher than in the group of normal subjects.

The level of serum iron and index of transferrin saturation do not always anticipate the amount of iron excretion after DF The correlation between serum iron and urinary iron excretion was only 0.57 while the correlation between percentage of transferrin saturation and

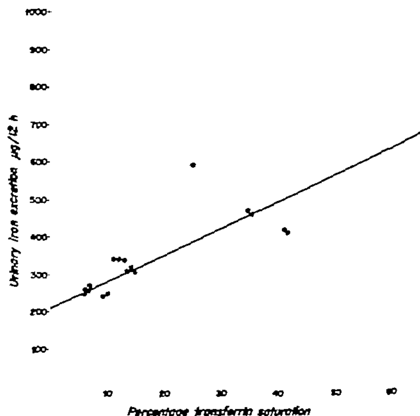


Fig 2. Correlation between the urinary iron excretion and the percentage of transferrin saturation.

urinary iron was somewhat higher 0.43 (fig 2) WALSH *et al.* [9] have found that the percentage of transferrin saturation was satisfactory to predict the outcome of the DF test. It appears that in our cases however the amount of stainable iron in the bone marrow was more reliable to estimate the excretion of iron

Summary

The 12 hour urinary excretion of iron after an intravenous injection of 500 mg of desferrioxamine was measured in apparently normal subjects and patients with low serum iron with or without depleted iron stores and anemia. The iron excretion was significantly higher in patients with normal or high iron stores than in patients with iron deficiency anemia. Overlapping was observed between the values of urinary iron of normal women and patients with iron deficiency anemia. The urinary iron was lower in apparently hematologically normal women than in males. The results seem to indicate that desferrioxamine could be used to differentiate anemias with low serum iron due to chronic infections, carcinomas, and similar conditions from iron deficiency anemias.

Acknowledgement

We are indebted to Dr. ALFREDO CORONEL, Dr GUSTAVO GARCIA-GALINDO and Dr HERMOGENES RIVERO for permission to study some cases hospitalized in their wards, and to Dr JACK FIELDING for this suggestions.

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On the Platelet Levels in Chronic Iron Deficiency Anemia[1]

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Until recently the platelet levels in chronic iron deficiency anemia were considered to vary in normal ranges, even showing a slight decrease in some cases of long duration [1a] With the publications of Schloesser et al. [2] and Gross et al. [3] where the association of thrombocytosis with iron deficiency anemia have been described, a tendency seems to have grown to consider the platelet level in this disorder as increased [1b, 4] Therefore, we have regarded it worthwhile to re-evaluate this finding in 39 patients with different types of chronic iron deficiency anemia.

Material

Patients with iron deficiency anemia were selected only when the following criteria were satisfied

1 Hematologic criteria of iron deficiency anemia such as hypochromia and microcytosis in the stained peripheral blood film, low mean corpuscular hemoglobin and decreased mean corpuscular hemoglobin concentration.

2. A hemoglobin level less than 10.5 gm%.

3. A serum iron level less than 80 μg%.

4 The correction of the anemia by appropriate replacement therapy Cases other than pure iron deficiency anemia were excluded from the study Patients in whom iron deficiency anemia had developed during the course of neoplastic disorder or chronic infection, and patients with either manifest or occult active blood loss were also excluded. Only those whose blood loss was due to an unique cause like hemorrhoids or menstruation, were admitted on the condition that the last bleeding had stopped at least 3 weeks ago. Five patients with chronic iron deficiency anemia who had active blood loss during their admission were

[1] This paper was presented at the XII Congress, International Society of Hematology New York, September 3, 1968.

excluded from the main group of patients and were studied separately together with a group of 4 patients with chronic iron deficiency anemia who had been given iron outside the hospital.

Ten patients with chronic iron deficiency anemia associated with hypersplenism, and 29 patients with uncomplicated chronic iron deficiency anemia formed the main group of study.

Methods

The hematological methods were all standard technics. Platelet counts were performed with phase contrast microscope using the Brecher-Cronkite method [5]. Serum iron determinations were performed by the method of Seales [6] with a Unicam spectrophotometer. In appropriate cases d-xylose absorption test was performed [7]. Fetal hemoglobin was estimated by the method of Singer *et al.* [8]. Methods of Huisman and Dozy [9] and Aksoy and Erdem [10] using DEAE-cellulose chromatography and starch gel electrophoresis were applied for the quantitation of hemoglobin A_2 in appropriate cases. All other biochemical investigations were carried out by routine laboratory technics.

Therapy consisted of oral administration of reduced iron 1.5 g per day except in cases with defective intestinal absorption, to whom iron was administered parenterally as 100 mg iron-sorbitol-citrate complex every other day. Patients with hypersplenism were submitted to splenectomy.

Results

For purposes of comparison of the initial platelet levels, the 39 patients were divided into two groups.

Group 1 consisted of 10 patients with severe chronic iron deficiency anemia, and hypersplenism, with splenomegaly and pancytopenia which was due to portal hypertension of long duration, mostly originating in early childhood from insufficient intake of animal proteins. This group of patients were named cases of chronic iron deficiency anemia associated with hypersplenism (IDA+ HS). The average initial platelet counts ranged, between 60.5 and $82 \times 10^3/mm^3$ with an average of $72 \times 10^3/mm^3$. Standard deviation (SD) was $\pm$ 26 and the p value less than 0.02. The hematologic data of this group are summarized in table I.

Group 2 comprised 29 patients with uncomplicated iron deficiency anemia (IDA). The etiological factor in most of the cases was malnutrition (Mn), others being chronic blood loss (CBL), parasitic infestation (P), malabsorption (Ma), endocrine dysfunction (E) (Sheehan's syndrome) and various combinations of these. The average initial platelet level in this group was $270 \times 10^3/mm^3$, the range being 121.5-

Table I Hematologic data in 10 patients with chronic iron deficiency anemia associated with hypersplenism

No.	Sex	Hb g%	PVC %	RBC X10⁶/mm	Reti. %	MCV µm	MCH pg	MCHC %	Ser F µg%	WBC /mm	Plats. ×10⁴/mm	Hypo-chromia
1	F	3.9	22	2.84	0.5	78.3	21	26.8	65	2,800	66.0	3+
2	F	3.6	23	3.18	0.1	74.0	18	24.3	56	1,100	63.0	3+
3	F	3.7	26	3.30	0.4	78.7	17	21.9	36	2,800	70.0	3+
4	F	2.4	10	1.37	0.6	76.8	18.3	24.0	65	1,400	72.5	4+
5	F	2.0	9.5	2.40	0.4	39.3	8.7	21.0	57	2,200	80.0	4+
6	F	4.6	19	2.60	2.0	73.0	17.7	25.0	65	2,300	65.0	4+
7	F	6.2	19	2.72	0.1	70.3	22.9	32.7	72	2,000	60.5	3+
8	M	4.8	18	3.00	1.0	60.0	13.0	25.0	39	1,200	81.0	4+
9	F	4.2	18	2.50	2.0	72.0	16.0	23.0	30	2,800	82.0	3+
10	F	3.9	22	2.90	0.2	73.8	20.3	26.7	55	2,000	80.0	3+

Table II Hematologic data in 29 patients with uncomplicated iron deficiency anemia

No.	Sex	Etiology*	Hb g%	PCV %	RBC ×10⁶/mm³	Retic %	MCV µm³	MCH pg	MCHC %	Ser.F µg %	WBC /mm³	Plats ×10³/mm³	Hypochromia
1	F	Mn+G	4.9	20	2.70	0.5	74	18	24.5	78	5,600	415	3+
2	F	Mn	4.2	22	3.19	0.4	68	13	19.0	45	6,400	305	4+
3	F	CBL	4.3	23	2.96	0.7	79	14.4	18.6	75	9,400	228	4+
4	F	Mn+G	5.9	26	3.54	0.1	72.2	16.6	22.6	70	5,400	272	4+
5	F	CBL	8.0	27	3.49	0.2	79	23	29.8	36	7,900	210.5	3+
6	F	Mn	5.6	20	2.81	0.8	71.4	20	28.0	46	9,200	145	3+
7	M	CBL	5.1	18	2.30	0.2	78.2	22.1	28.3	38	6,800	340	3+
8	M	CBL	5.2	20	2.60	1.0	73	20	26.0	50	3,800	225	3+
9	M	CBL	3.6	13	1.71	1.0	76	21.1	27.0	35	3,200	225	4+
10	F	E	9.1	33	4.21	0.2	78.5	21.6	27.5	52	3,000	278	4+
11	M	Mn	2.5	15	2.20	0.1	68	11.3	16.6	35	8,600	195	4+
12	F	Mn	8.7	19	3.85	0.6	76.3	22.6	30.0	46	4,800	247.5	3+
13	M	Mn	9.3	33	4.38	1.0	76.7	21.6	28.1	56	4,600	287.5	3+
14	F	Mn	8.5	27	3.62	0.8	75	23.6	31.4	75	6,200	330	3+
15	M	M	10.2	32	4.12	0.8	78	24.8	31.9	56	8,800	357	2+
16	M	P	9.1	34	4.31	0.2	79	21.1	26.7	62	5,600	290	2+
17	M	Mn+P	4.8	20	2.70	2.0	74	17.7	24.0	25	4,400	340	4+
18	M	M	9.8	30	3.81	0.1	78.9	23.7	32.6	75	7,400	370	2+
19	F	M +CBL	6.6	23	2.97	0.4	79.3	22.7	28.6	63	3,200	137	3+
20	M	Mn+P	3.0	19	2.42	2.0	75	12.3	15.7	42	3,000	360	4+
21	F	Mn+P	8.7	30	3.92	0.1	76.9	22.3	28.3	35	5,200	300	4+
22	F	Mn	5.7	19	2.94	0.4	65.5	19.6	30.0	64	8,000	190	4+
23	F	Mn	6.6	24	3.33	0.8	72.7	20	27.5	68	5,200	350	3+
24	M	Mn+P	7.7	30	3.85	1.0	78.9	20.2	25.6	74	7,300	290	3+
25	M	Mn+CBL	6.2	22	3.46	1.0	64.3	18.2	28.1	43	8,400	265	4+
26	F	Mn+G	7.0	24	3.33	0.8	72.7	21.2	29.1	60	6,200	200	3+
27	M	Mn	6.0	22.5	2.93	0.6	77.5	20.6	26.6	48	5,800	184	3+
28	M	Mn	6.5	20	2.64	0.4	78.9	25	32.5	36	4,800	121.5	4+
29	F	Mn	7.3	23	3.40	1.0	73.5	21.4	29.2	62	6,600	370	3+

Mn Malnutrition, *G* Gastrectomy *CBL* Chronic blood loss, *E* Endocrine defect, *M* Malabsorption, *P* Parasitic infestation

$415 \times 10^3/mm^3$ Standard deviation was $\pm$ 137 and the p value was less than 0 02. The initial hematologic data of this group are presented in table II

We have also studied separately another group of patients, designated as group 3 which consisted of 9 patients, 5 of whom had active blood loss at the time of admission, and 4 had received iron treatment outside the hospital In patients with active blood loss the average values were as follows hemoglobin 6 1 g % serum iron 46 μg % platelets $574 \times 10^3/mm^3$ For those who received iron therapy outside, these average values were hemoblobin 6.4 g%, serum iron 52 μg% platelets $528 \times 10^3/mm^3$ In this group the initial platelet counts were higher than the upper limit of the BRECHER CRONKITE method [10]

The initial average values of platelets of 15 healthy subjects are given in table III Standard deviation for the control group was $\pm$ 24 and the p value less than 0.02. In all patients of group 1 and in 7 patients of group 2, we had the opportunity to study the platelet levels after the initiation of iron treatment. On the third or fourth day a rise in the platelet counts was observed, the average being 69 % (SD $\pm$ 47) for group 1 and 53% (SD $\pm$ 108) for group 2. All p values were less than 0 02.

In all cases submitted to splenectomy a permanent rise in the platelet levels was noted with the correction of hypersplenism.

Discussion

As can be seen from our results, the initial platelet counts of iron deficient patients are distributed over a wide range, the mean being very low in the first group when compared with that of the second, being an expected result. This is due to the pancytopenia resulting from secondary hypersplenism present in all the patients of the first group and therefore seems to be different in origin from the thrombocytopenia detected by GROSS *et al.* [3] As leukocyte counts of their iron deficient patients, are not reported, the presence of hypersplenism is difficult to determine. Thus, our first group remains a separate entity in whom chronic iron deficiency was associated with hypersplenism. All the patients in this group had splenomegaly or hepatosplenomegaly chronic iron deficiency anemia and, besides, 5 had infantilism and hypogonadism. This corresponds to the syndrome described by TAYANÇ

Table III. Sex, age distribution and initial average values of hemoglobin, serum iron and platelets in 15 normal subjects (N) 29 patients with iron deficiency anemia (IDA) and 10 patients with chronic iron deficiency anemia associated with hypersplenism (IDA + HS)

Group	No	Females	Males	Mean age years	Mean Hb g%	Mean serum iron μg%	Mean platelet count /mm
N	15	6	9	25	12.3	96.6	204 000 SD ± 24,000
IDA	29	16	13	30	6.7	56.5	270,000 SD ± 157 000
IDA + HS	10	9	1	24	4 7	32.0	72,000 SD ± 26,000

p values were less than 0.02 for mean differences between the platelet counts of normal subjects and iron deficient patients with and without hypersplenism.

[11] later by REIMANN [12] PRASAD *et al.* [13] and recently by AKSOY *et al.* [14] The cause of the splenomegaly in 7 patients with chronic iron deficiency anemia was portal hypertension, possibly produced by the deleterious effect of malnutrition on the liver due to the inadequate intake of animal proteins since early childhood [14] Malnutrition was also the cause of iron deficiency anemia. Two patients had the pica of clay eating which diminished per se the intestinal absorption of the little amount of iron that could perhaps be taken by chance [15] In the remaining 3 patients, splenomegaly was due to chronic hepatitis or compensated cirrhosis of the liver No patient had the clinical and laboratory evidence of active blood loss.

The second group under study was the main subject of interest. The patients were cases of uncomplicated iron deficiency anemia, the etiologic factor being mainly malnutrition besides other causes present ed in table II.

Contrary to the findings of SCHLOESSER *et al.* [2] and GROSS *et al.* [3] none of the patients in this group had high platelet counts. The initial values all ranged in normal limits of the method employed [5] whereas, the platelet counts of the patients, studied by the above cited authors were at levels worthy to be denoted as thrombocytosis. Another important fact is the presence of active blood loss in their series, especially that of SCHLOESSER *et al.* [2] which comprised of female patients with menorrhagia, and the rest with hiatal hernia and gastrointestinal bleeding As denoted in the article, 38 of the 46 patients had active blood loss at the time of the investigation. Thus, increased platelet counts seem to go parallel with active blood loss, in the absence of which normal values are obtained. This was confirmed by the results of our third group studied separately for purposes of comparison. In this group patients had active blood loss at the time of admission and, as an evidence of this, had reticulocytosis (mean 2.6 %) and 4+ stool benzidine reactions. The platelet counts were significantly higher than the upper limit of the BRECHER-CRONKITE method the mean value being 574×10 /mm was similar to those obtained by SCHLOESSER *et al.* [2]

In 17 patients, *10 with iron deficiency anemia associated with* hypersplenism and 7 with uncomplicated iron deficiency anemia, the initiation of iron therapy produced a transient rise in the platelet levels which attained normal values even in those patients with hypersplenism. The platelets began to rise steadily approximately on the third or

fourth day after iron was started and later returned to previous levels. Although no attempt was made to detect the exact time the platelet counts returned to normal levels, they usually decreased to previous levels within a period of one month. Contrary to this, in a case of uncomplicated iron deficiency anemia belonging to group 2, the platelet count was still found to be $637.5 \times 10^3/mm^3$ one month after the initiation of oral iron therapy. Two months later the platelet level of this patient was $302 \times 10^3/mm^3$. A similar result was obtained with oral iron in a case of group 1 in whom the platelets rose from 70 to $955 \times 10^3/mm^3$ in a period of 2 weeks, falling to $240 \times 10^3/mm^3$ at the end of one month. Thus, in cases with pancytopenia, oral iron had no beneficial effect on the thrombocytopenia, but soleley corrected the anemia. In cases with uncomplicated iron deficiency anemia, the iron initiated rise in the platelets was 53% and in the group with hypersplenism, 69%, being statistically significant.

In cases submitted to splenectomy a permanent rise in the platelet levels was observed. With the exception of one case all 5 patients had high counts at the postoperative period. The one case cited above died with haemorrhage. The platelet count of this case was never normal.

The results obtained by OLEF [16], SCHLOESSER *et al.* [2] and GROSS *et al.* [3] are entirely different from those of our investigation. For the explanation of this difference concerning the platelet levels in chronic iron deficiency anemia, further studies are needed. Patients with chronic iron deficiency anemia who will be subjects to platelet studies should not receive any anti anemic agent and must not have experienced blood loss at least for a period of 3 months.

Thus, we are of the opinion that, in iron deficiency anemia, platelets are at normal levels unless the situation is complicated either with hypersplenism causing thrombocytopenia, or with active blood loss, producing thrombocytosis.

Summary

In 39 patients with various forms of chronic iron deficiency anemia, platelet levels were determined. Patients with occult or manifest bleedings were excluded. Contrary to the results of some authors, in no patient the initial platelet count was higher than normal. Cases with splenomegaly had low values due to hypersplenism. After iron therapy, the platelet number rised, even in cases with hypersplenism. In case of dietary deficiency thrombocytosis persisted for about 5 weeks.

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Lymphozytentransformationstest bei chronischer lymphatischer Leukose unter Berücksichtigung der absoluten Lymphozytenzahl im Blut

K. M. Heine, H. Stobbe, E. Hofer und H. Weber

Lymphozyten gesunder Personen haben die Fähigkeit, sich nach Antigenstimulierung zu teilungsfähigen Blasten, sog. Immunoblasten, umzuwandeln. Neben spezifischen Antigenen wie z.B. Streptolysin O (SLO) und Tuberkulin (PPD), die nur bei sensibilisierten Spendern lymphozytenstimulierend wirken, gibt es unspezifisch wirksame Stoffe, von denen der gebräuchlichste Phytohämagglutinin (PHA) ist. Dieses führt unabhängig von dem immunologischen Status des Lymphozytenspenders zur Lymphozytentransformation und ist daher für allgemeine Funktionsteste geeignet.

Für die chronische lymphatische Leukose (CLL) wurde eine herabgesetzte Transformationsfähigkeit der Lymphozyten von verschiedenen Autoren beschrieben [2, 5, 9, 11, 13, 14, 15, 17, 18, 19, 20, 21, 22, 23, 24, 25]. Aufgrund dieser Befunde entwickelte Dameshek [7] die Hypothese, dass der CLL eine Akkumulation eines möglicherweise schon genetisch determinierten immunologisch inkompetenten Lymphozytenklons zugrunde liege. In Folge der Funktionsuntüchtigkeit kommen diese Lymphozyten im Organismus nicht zur Verwendung, *überaltern, und es kommt dadurch zu ihrer zunehmenden* Anhäufung. Die genannten Autoren berücksichtigten jedoch bei ihrer Auswertung nur die prozentualen Anteile transformierter Lymphozyten in der Kultur, ohne diese in Beziehung zu den Lymphozytenwerten in der Volumeneinheit Blut zu setzen. Die Berücksichtigung der Absolutwerte hat jedoch unzweifelhaft den Vorrang vor den Relativwerten, durch die Fehleinschätzungen zustande kommen.

In der vorliegenden Arbeit soll am Beispiel der CLL versucht werden, durch Betrachtung der prozentualen Lymphozytentransformationswerte im Zusammenhang mit den absoluten Lymphozytenwerten des Blutes, die Funktionstüchtigkeit des lymphatischen Systems besser als bisher einzuschätzen. Darüber hinaus soll zu der Frage Stellung genommen werden, ob es sich entsprechend der Theorie von DAMESHEK [7] bei der CLL um ein Mosaik einer gesunden und einer pathologischen Lymphozytenpopulation handelt, welche schließlich die gesunde verdrängt, oder ob eine einheitliche Zellpopulation vorliegt.

Material und Methode

Es wurden Lymphozytenkulturen von 26 unbehandelten Patienten mit leukämisch verlaufender CLL im Alter von 53–79 Jahren untersucht.

13 Patienten wurden unmittelbar nach Durchführung einer spezifischen Therapie kontrolliert, und bei 7 Patienten wurden wiederholte Kontrolluntersuchungen vor und nach Therapie angestellt. Die Behandlung bestand dreimal in einer Röntgenbestrahlung der Milz mit einer Gesamtdosis von 200–300 bei wöchentlicher Verabfolgung von 50 8mal in Gaben von Cyclophosphamid in einer Gesamtdosis von 2,2–8 g bei oraler Einnahme von 100–200 mg täglich und 2mal in Gaben von Prednison in einer Gesamtdosis von 0,5 mg. Als Vergleichsgruppe dienten uns 55 gesunde Probanden im Alter zwischen 19 und 60 Jahren.

Die Lymphozyten wurden aus heparinisiertem Venenblut durch Sedimentierung der Erythrozyten gewonnen und auf eine Endkonzentration von 1,2 × 10^6/ml Kulturflüssigkeit eingestellt.

Die Lymphozytenstimulierung erfolgte parallel mit PHA, SLO und PPD. Die Züchtung wurde in Eagle MEM Zellzüchtungsmedium[4] unter Zusatz von Penicillin und Streptomycin sowie 20% autologem Plasma oder hitzeinaktiviertem Human-AB-Serum durchgeführt. Die PHA-stimulierten Kulturen wurden nach 72 h, die SLO- und PPD-stimulierten nach 120 h abgebrochen. Die Auswertung erfolgte durch Differenzierung von 1000 lymphatischen Zellen in transformierte und nichttransformierte Lymphozyten im panoptisch gefärbten Objektträgerausstrich. Aus den so erhaltenen Prozentwerten und den absoluten Lymphozytenzahlen in der Volumeneinheit des peripheren Blutes wurde die Zahl transformierter Lymphozyten/µl Blut berechnet.

Da die Transformationshäufigkeiten in allen 3 Gruppen eine sehr schiefe, teilweise fast J-förmige Verteilung aufwiesen, wurde zur statistischen Prüfung der Rangsummentest nach WILCOXON, bei den PPD-Werten der Mediantest benutzt.

Ergebnisse

Die Mittelwerte der Lymphozytentransformationsquoten bei Gesunden und Patienten mit CLL vor und nach Therapie sowie der statisti

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Tabelle I. Mittlere Lymphozytentransformation bei Gesunden und Patienten mit CLL (Übersicht und statistische Ergebnisse)

	Lymphozytentransformation nach Stimulierung mit PHA	SLO	PPD
	$\bar{x}$	$\bar{x}$	$\bar{x}$
a) Prozentuale Transformationswerte			
Normgruppe	82	34	15
	$p < 0,001$	$p < 0,001$	$p < 0,001$
CLL unbehandelt	1	6	3
	$p < 0,001$	$p < 0,001$	$p < 0,001$
CLL nach Therapie	40	34	20
	$p < 0,001$	$p > 0,05$	$p > 0,05$
Normgruppe	82	34	15
b) Absolute Transformationswerte			
Normgruppe	1490	645	290
	$p > 0,05$	$p > 0,05$	$p > 0,05$
CLL unbehandelt	4460	1720	370
	$p > 0,05$	$p < 0,01$	$p < 0,01$
CLL nach Therapie	6500	2800	1250
	$p < 0,001$	$p > 0,05$	$p > 0,05$
Normgruppe	1490	645	290

sche Vergleich der Unterschiede sind in Tabelle I zusammengestellt. Die Einzelwerte zeigen die Abbildungen 1 und 2. Die Transformationsquoten (Mittelwerte und Variationsbreiten) betrugen bei Gesunden nach Stimulierung mit PHA 82% (51–94%), SLO 34% (1–89%), PPD 15% (0–68%). Demgegenüber waren die Werte bei Patienten mit einer unbehandelten CLL deutlich herabgesetzt und betrugen nach Stimulierung mit PHA 12% (0–47%), SLO 6% (0–47%), PPD 3 (0–53). Der Unterschied zwischen der Normgruppe und der CLL-Gruppe war für alle Werte mit einer Irrtumswahrscheinlichkeit von $p < 0{,}001$ signifikant.

Berechnet man aus der absoluten Lymphozytenzahl im Blut und dem prozentualen Anteil transformierter Lymphozyten in der Kultur die absolute Zahl transformierter Lymphozyten/µl Blut, so ergeben sich für die Gesunden nach Stimulierung mit PHA 1490/µl (810–2380/µl), SLO 645/µl (12–2070/µl), PPD 290/µl (0–1900/µl). Die CLL-Gruppe wies dagegen etwas höhere Werte auf. Sie betrugen nach Stimulierung mit PHA 4460/µl (0–22000/µl), SLO 1720/µl (0–20 00/µl), PPD 370/µl (0–2600/µl). Die Unterschiede gegenüber der Normgruppe waren statistisch nicht signifikant (Tab. I).



Abb. 1 Prozentuale Lymphozytentransformation nach Stimulierung mit PHA, SLO und PPD bei unbehandelten (a) und behandelten (b) Patienten mit chronischer lymphatischer Leukose im Vergleich zu einer gesunden Kontrollgruppe ( )

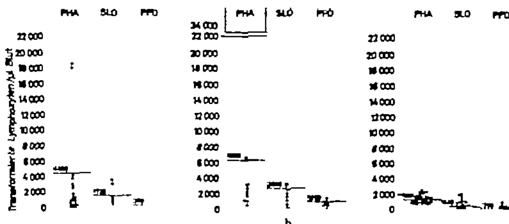


Abb. 2. Absolute Lymphozytentransformationswerte nach Stimulierung mit PHA, SLO und PPD bei unbehandelten (a) und behandelten (b) Patienten mit chronischer lymphatischer Leukose im Vergleich zu einer gesunden Kontrollgruppe (c).

Nach Durchführung einer spezifischen Therapie kam es bei den Patienten mit CLL zu einem deutlichen Anstieg des prozentualen Anteils transformierter Lymphozyten in der Kultur (Abb. 1) nach Stimulierung mit PHA 40% (13–81%) SLO 34% (3–84%) PPD 20% (1–85%) Die Abbildungen 3–9 illustrieren die Auswirkungen einer Behandlung auf die absoluten und relativen Transformations-

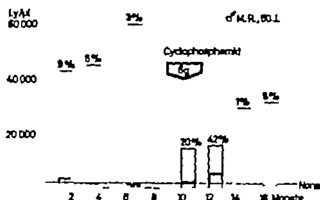


Abb. 3. Einfluß einer oralen Cyclophosphamidbehandlung auf die Lymphozytentransformation bei chronischer lymphatischer Leukose nach Stimulierung mit PHA. Während die prozentualen Transformationswerte um das Mehrfache ansteigen, zeigen die absoluten Transformationswerte keine nennenswerten Änderungen.

Schraffierte Säulen = Transformationswerte/µl Blut. Weiße Säulen = *Lymphozytenwerte/µl Blut. Der Normbereich der Lymphozytentransformation bei Gesunden ist durch* gestrichelte Linien gekennzeichnet. Die Prozentzahlen beziehen sich auf die Transformationswerte/µl Blut (schraffierte Säulen)

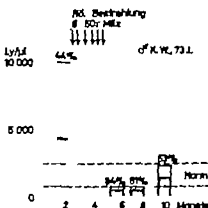


Abb. 4

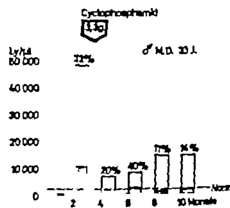


Abb. 5

Abb. 4 und *5.* Einfluß einer Röntgenbestrahlung der Milz bzw. einer Cyclophosphamidbehandlung auf die Lymphozytentransformation bei chronischer lymphatischer Leukose *nach Stimulierung mit PHA. In beiden Fällen kommt es zu einem* Absinken der absoluten Transformationswerte bei Anstieg der Prozentwerte (Legende siehe Abb. 3).

werte bei Patienten mit chronischer lymphatischer Leukose nach Stimulierung mit PHA. Sie zeigen, daß die Hauptschwankungen bei den nicht transformierten Lymphozyten auftreten. Die Angabe der Lymphozytentransformation in Prozentwerten täuscht einen stärkeren

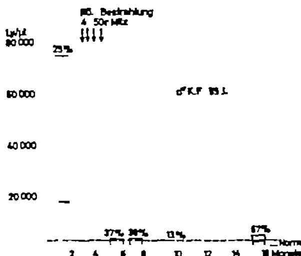


Abb. 6. Einfluss einer Röntgenbestrahlung der Milz auf die Lymphozytentransformationswerte bei chronischer lymphatischer Leukose nach Stimulierung mit PHA. Trotz deutlichem Anstieg der Prozentwerte kommt es zu einer Reduktion der absoluten Transformationswerte (Legende siehe Abb. 3)

Einfluss der Therapie auf die Transformationswerte vor, als es der Realität entspricht.

Dem deutlichen, auch statistisch mit $p < 0.001$ signifikanten Anstieg (Tab. I) entsprach ein im Mittel weniger ausgeprägter Anstieg der absoluten Zahl transformierter Lymphozyten, und im Einzelfall kam es sogar häufig zu einem Abfall der Werte (Abb. 4, 5, 6). Die absoluten Transformationswerte nach Therapie betrugen nach Stimulierung mit PHA 6500/µl (648–34200/µl), SLO 2800/µl (240–10260/µl), PPD 1250/µl (42–8550/µl). Der Anstieg gegenüber der Untersuchung vor Therapie war nur für die SLO- und PPD-stimulierten Kulturen mit einer Irrtumswahrscheinlichkeit von $p < 0.01$ signifikant (Tab. I).

Die Ergebnisse heissen in erster Linie an einen Zusammenhang zwischen der Lymphozytenzahl im Blut und dem Prozentsatz transformierter Lymphozyten denken, denn unter der Therapie kommt es im allgemeinen zu einem Abfall der Lymphozytenwerte im Blut. Aus diesem Grunde wurde die Korrelation zwischen den Logarithmen der Lymphozytenzahl/µl Blut und den prozentualen Transformationswerten nach PHA-Stimulierung bei unbehandelten CLL-Patienten überprüft. Es konnte eine negative Korrelation beider Grössen gesichert

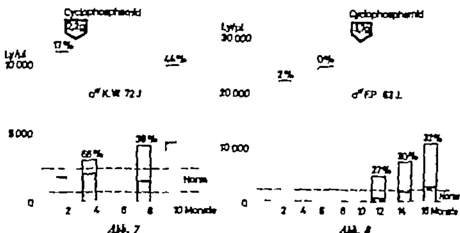


Abb. 7 *Abb. 8*

Abb. 7 und *8*. Einfluss einer oralen Cyclophosphamidbehandlung auf die Lymphozytentransformation bei chronischer lymphatischer Leukose nach Stimulierung mit PHA. In beiden Fällen steigen die absoluten und relativen Transformationswerte an (Legende siehe Abb. 5)

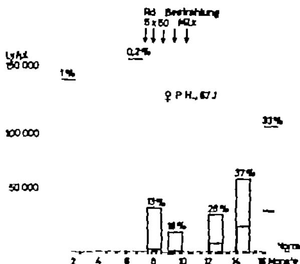


Abb. 9 Einfluss einer Röntgenbestrahlung der Milz auf die Lymphozytentransformation bei chronischer lymphatischer Leukose nach Stimulierung mit PHA. Es kommt zu einem Ansteigen der absoluten und relativen Transformationswerte (Legende siehe Abb. 5).

werden ($p < 0{,}001$) d.h. die prozentualen Transformationswerte sinken mit steigender Lymphozytenzahl. Dagegen bestand kein Zusammenhang zwischen der Lymphozytenzahl im Blut und der absoluten Zahl transformierter Lymphozyten. Dies besagt, dass mit steigender Lymphozytenzahl die immunologisch insuffizienten Lymphozyten re-

lativ stärker zunehmen als die noch transformationsfähigen. Eine Abnahme der immunologisch kompetenten, durch Antigen stimulierbaren Lymphozyten mit steigenden Lymphozytenwerten, konnten wir dagegen nicht feststellen.

Diskussion

In Übereinstimmung mit den in der Literatur mitgeteilten herabgesetzten Lymphozytentransformationswerten war auch bei den von uns untersuchten Patienten mit CLL der prozentuale Anteil transformierter Lymphozyten nach Stimulierung mit PHA, SLO und PPD deutlich vermindert. Die Berechnung der absoluten Werte führte jedoch zu einem ganz anderen Bild. Die Zahl transformierter Lymphozyten war im Mittel nicht nur nicht herabgesetzt, sondern sogar gegenüber der Norm erhöht. Der Mittelwert sagt hier allerdings weniger aus als die Betrachtung der Einzelwerte (Abb. 2) Diese zeigen, dass ein Grossteil der Werte im Normbereich, ein kleinerer Teil darunter und ein weiterer Teil wesentlich über der Norm lag Dies gilt sowohl für die Stimulierung mit PHA als auch für die Stimulierung mit den spezifischen Antigenen SLO und PPD

Die Tatsache, dass die transformierten Lymphozyten in manchen Fällen gegenüber der Norm vermehrt sein können, spricht gegen die Hypothese der Anhäufung eines von vornherein immunologisch inkompetenten Zellklons. Es sei denn, man nimmt an, dass in Folge der Vermehrung pathologischer für den Organismus als fremd erkennbarer Lymphozyten die Abwehrleistung des verbliebenen normalen lymphatischen Gewebes so stimuliert wird, dass es zu einer reaktiven Vermehrung der funktionstüchtigen Zellen kommt. Da man den Lymphozyten u.a. auch die Funktion der Tumorabwehr im Sinne einer host-versus-graft Reaktion zuschreibt [6, 8 10, 12 16] wäre dies durchaus vorstellbar Erst im Spätstadium, wenn das normale lymphatische Gewebe mehr und mehr verdrängt wird, käme es dann zu einer absoluten Abnahme immunologisch vollwertiger Lymphozyten. Diese Verminderung war in einzelnen Fällen tatsächlich nachweisbar Sie kann aber ebenso durch das Vorliegen einer einheitlichen Lymphozytenpopulation erklärt werden, innerhalb welcher es zu einem fortschreitenden Verlust der Transformationsfähigkeit kommt.

Über den Einfluss einer spezifischen Therapie auf die Lymphozytentransformation bei Patienten mit CLL besteht in der Literatur keine einheitliche Meinung [1 3 4 19 24 25] Astaldi [3] fand eine Ver

besserung der prozentualen Transformationswerte nach einer Röntgenbestrahlung der Milz. Andere Autoren führten lediglich eine Unterteilung ihrer Patienten in behandelte und unbehandelte Fälle durch, ohne den Zeitpunkt der Therapie zu berücksichtigen [4 19 24, 25] und kamen zu widersprüchlichen Ergebnissen. Uns scheint auch hier die Berücksichtigung der Lymphozytenzahl des peripheren Blutes wesentlich, denn wie aus den Abbildungen 3–9 hervorgeht, täuscht ein Anstieg der prozentualen Werte transformierter Lymphozyten oft über eine tatsächliche Verminderung derselben hinweg Aus unseren Untersuchungen an zytostatisch behandelten und Milz bestrahlten Patienten geht hervor dass es unter der Therapie zu einer Reduzierung vorwiegend der nicht stimulierbaren Lymphozyten, jedoch oft auch zu einem Rückgang der noch funktionstüchtigen Lymphozyten kommt. Die negative Bedeutung für die in ihrer Abwehr ohnehin schwer geschädigten Patienten liegt auf der Hand.

Zusammenfassung

Bei der chronischen lymphatischen Leukose ist der prozentuale Anteil transformationsfähiger Lymphozyten deutlich vermindert. Dies beruht auf einer Vermehrung funktionsuntüchtiger d.h. durch Antigen (Phytohämagglutinin, Streptolysin O und Tuberkulin) nicht stimulierbarer Lymphozyten. Es kommt jedoch in den meisten Fällen nicht zu einer Verminderung der absoluten Zahl transformierbarer Lymphozyten. Die Anzahl transformierter Lymphozyten/µl Blut ist häufig normal, mitunter sogar wesentlich gegenüber der Norm erhöht. Letztere Fälle sind insofern von besonderem Interesse, als sie der Theorie einer reinen Akkumulation eines immunologisch inkompetenten Lymphozytenklons als Ursache für die Lymphozytenvermehrung bei der CLL widersprechen. Nach Durchführung einer zytostatischen Behandlung oder einer Röntgenbestrahlung der Milz kommt es zu einer Lymphozytensenkung im Blut, vorwiegend auf Kosten der nicht stimulierbaren Lymphozyten, und damit zu einer relativen Vermehrung der funktionstüchtigen Lymphozyten. Diese Verbesserung der relativen Transformationswerte täuscht jedoch oft über einen tatsächlichen Abfall der absoluten Zahl funktionstüchtiger Lymphozyten hinweg.

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Enhancement of the Fibrinolytic Activity by Venous Occlusion in Patients with Primary 'Carbohydrate-Induced' Hypertriglyceridemia

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Venous occlusion is reported to produce a marked increase in fibrinolytic activity in the blood [1 3, 9 17 20, 21 25] Postocclusional release of plasminogen activator (PA) – probably from the vasa vasorum [17 21 25] – seems to counteract hypercoagulability due to stasis, ischemia, concentration of plasma proteins and activation of the intrinsic clotting system [12] In patients with venous thrombosis Pandolfi et al [25] demonstrated a decreased PA content of the vessel walls and a weak enhancing of fibrinolytic activity by venous occlusion.

Experimental venous occlusion seems to be a suitable model to study the vessel response to the thrombogenic influences mentioned above. Patients with primary carbohydrate induced hypertriglyceridemia (PCH) have a high percentage of cardiovascular and occlusive arterial disease [7] Apart from a direct effect of the increased plasma lipids on the arterial wall an inhibition of the fibrinolytic system in these patients has also to be taken into consideration as a cause of thrombus formation and probably also as a cause of atherogenesis [24 28 31] In previous papers concerning the fibrinolytic system in patients with PCH we have reported on an increase of fibrinogen, proactivator plasminogen slow-reacting antiplasmin and in the inhibitors of plasminogen activation by urokinase, whereas PA activity was found to be decreased [28 29 30] The inhibition of PA release from the vessel wall is supposed by Howell [18] to be one of the points, where the plasminogen activation sequence is blocked by lipids. The

purpose of the present study was to investigate the PA release following venous occlusion in patients with PCH

Material and Methods

Twenty-four normal persons ranging in age from 23 to 50 years with triglyceride levels not over 160 mg% and 29 patients with PCH, ranging in ge from 36 to 71 years, with tri-

Table I

Pat.	Age years	Sex	Trigl. mg%	Chol. mg%	FFA mEq/l	PL mg%	Pre-β	Activator mm² pre- occlusion	post occlusion
S.R.	50	m	8930	2930	1060	1730	+++	28	20
H.E.	36	m	4670	1390	900	1240	+++	7	56
M.F	68	m	2090	785	640	710	+++	6	18
H.R.	51	m	1230	630	580	660	+++	21	107
R.A.	37	m	1172	635	1140	610	+++	83	217
D.F	55	m	850	590	690	422	+++	20	216
S.E.	45	m	813	700	580	405	+++	63	237
M.E.	49	m	770	240	590	510	++	55	123
K.P	61	m	750	505	660	440	++	80	181
H.O.	70	m	730	471	710	435	++	6	29
S.K.	48	m	642	438	1150	375	++	36	132
B.H.	41	f	630	480	575	420	++	6	69
K.J.	34	m	520	275	550	345	++	87	125
S.R.	51	m	470	505	810	400	+++	6	56
H.O	34	m	460	325	670	335	+++	20	157
H.E.	36	m	372	346	1060	300	+++	25	102
G.L.	66	m	357	311	900	320	++	16	121
L.F	66	m	346	425	950	325	+	72	181
W.F	36	m	318	387	575	339	++	49	179
B.L.	47	m	307	385	415	294	++	85	268
R.H.	60	m	303	322	820	310	++	49	195
S.F	62	f	275	345	590	319	++	62	133
J.F	62	m	255	302	550	302	+++	25	209
E.H.	43	m	252	328	770	281	++	42	116
H.V	59	m	230	373	860	268	+	51	232
W.W.	64	m	222	495	660	363	+	27	58
S.A.	42	m	215	222	580	226	++	36	73
F.A.	61	m	205	295	650	257	++	53	290
M.A.	62	m	200	368	835	272	+	89	288
Mean			986	565	733	432		42	148

Trigl. triglycerides *Chol.* cholesterol *FFA* free fatty acids *PL* phospholipids; *Pre-β* semi-quantitative expression of the amount of pre-β-lipoproteins.

glyceride levels between 200 and 8,950 mg% were tested. All patients had a distinctly marked pre-β-band in the lipoproteinelectrophoresis (table I).

Blood was collected from the cubital vein by clear venepuncture in siliconized 96 × 25 mm glass tubes containing 3.8 mg dry trisodium citrat (Geigy Basle) per ml blood for fibrinolytic assay. For lipid assay 500 units of heparin (Novo Industri, Copenhagen) per 10 ml blood was added. The tubes were kept on ice and centrifuged immediately after withdrawal at +4°C for 10 min at 4,000 rpm.

Experimental procedure. The 'pre-occlusion sample' was drawn without the use of a tourniquet. Meanwhile a sphygmomanometric cuff was placed above the elbow of the other arm and inflated to a pressure midway between the systolic and diastolic one. After 10 min of venous occlusion the 'postocclusion sample' was taken.

Preparation of the euglobulin fraction. 0.5 ml of citrated plasma was added to 9.5 ml of distilled water and the pH adjusted to 5.6 with 1% acetic acid solution. The precipitated fraction was separated by centrifugation and redissolved in 0.5 ml tris-HCl-buffer pH 7.8, I = 0.15.

Plasminogen activator assay of the euglobulin fraction was performed by the fibrin plate method of Astrup and Müllertz [1] as modified by Nilsson and Olow [22, 23] using bovine fraction I (Armour Pharmaceutical Ltd., Eastbourne, England, batch No. MC 0670) and tris(-hydroxymethyl)-aminomethane-HCl-buffer pH 7.8. Activator activity was expressed as mm² of lysed area of the bovine fibrin film. Each test sample was run in triplicate and the arithmetic mean of the single determinations calculated. In preliminary experiments also free plasmin activity was measured. No free plasmin was found provided that the test system contained epsilon-aminocaproic acid in a concentration of 3×10^{-3} M.

Calculation of the amount of PA released by venous occlusion. For this purpose it was necessary to convert the mm² of lysed area to absolute values. A reference curve was calculated from serial dilutions of 3 highly active 'pre- and post-occlusion' euglobulin preparations. An arbitrary unitage was chosen: 10 arbitrary units (a.u.) = 300 mm² of lysed area under standard conditions (18 h of incubation at 37°C). Statistical evaluation shows two regression lines (fig. 1)

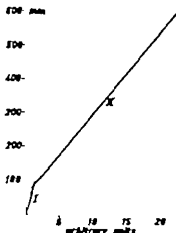


Fig. 1. Regression lines calculated from serial dilutions of three 'pre- and post-occlusion' euglobulin preparations. No. I ranging from 0–84 mm² (r = 0.5974, p = 0.03, y = 0.04 + 69.9X). No. II ranging from 84–635 mm² (r = 0.7624, p = 0.01, y = 57 + 24.4X).

No. I ranging from 0–84 mm² (= 0.5974 $p < 0.05$ N = 11
$y = 0.04 + 69.9$ X)
No. II ranging from 84–535 mm² (= 0.7624 $p < 0.01$ N = 10
$y = 37 + 24.4$ X).

The amount of PA released by venous occlusion was then calculated by converting the mm to arbitrary units and subtracting the 'pre-occlusion' activator from the post-occlusion activator. It should be mentioned that this method presents certain limitations especially at low activator values.

Lipids were assayed in the 'pre-occlusion' heparin plasma only. The plasma was extracted by CARLSON's [8] modification of the method of FOLCH *et al.* [13]. Lipid phosphorus was estimated according to BARTLETT [4] (lipid phosphorus × 25 = phospholipids), total cholesterol according to SEARCY *et al.* [27] and triglycerides according to CARLSON [8]. Free fatty acids (FFA) were titrated by the method of DOLE and MEINERTZ [11] as modified by TROUT *et al.* [32]. Lipoproteinelectrophoresis was performed according to FREDRICKSON and LEES [14].

Statistical analysis. Mean ($\bar{x}$) and standard deviation (sd) was calculated by the customary normal distribution methods. Normal distributed data were compared by Student's t test, otherwise Wilcoxon two sample test was used (S = sum of the ranks). The correlation coefficient ( ) was calculated by the formula

$$= \frac{S(x-\bar{x})\ (y-\bar{y})}{\sqrt{S(\ -\bar{x})^2\ \ S(y-\bar{y})^2}}$$

Results

1. Pre-occlusion activator level. Normal persons (N=24) $\bar{x}$ 101 mm² $s = \pm 43$ mm, patients with PCH (N=29) $\bar{x} = 42$ mm, $s = \pm 29$ mm² S=972, $p < 0.01$

2. Post-occlusion activator level. Normal persons $\bar{x} = 341$ mm² $s = \pm 126$ mm, patients with PCH $\bar{x} = 146$ mm² $s = \pm 81$ mm² S=940, $p < 0.01$

3. Activator released by venous occlusion. Normal persons $\bar{x} = 9.43$ a.u., $s = \pm 3.92$ a.u., patients with PCH $\bar{x} = 3.36$ a.u. $s = \pm 0.67$ a.u. $t = 8.22$ $p < 0.001$

4. In order to check the influence of the different lipid fractions on the pre-occlusion activator level and on the release of PA by venous occlusion, the correlation between triglycerides, cholesterol, phospholipids, FFA, and the pre-occlusion activator respectively the activator released by occlusion was calculated.

a) Correlation between triglycerides and	
pre-occlusion activator level	$r = -0.2714$ $p > 0.05$
activator released by occlusion	$r = -0.3693$ $p < 0.05$
b) Correlation between cholesterol and	
pre-occlusion activator level	$r = -0.2623$ $p > 0.05$
activator released by occlusion	$r = -0.3132$ $p > 0.05$

c) Correlation between phospholipids and
   pre-occlusion activator level $r = -0.3465,\ p > 0.05$
   activator released by occlusion $r = -0.3745,\ p < 0.05$
d) Correlation between FFA and
   pre-occlusion activator level $r = -0.1377\ \ p > 0.05$
   activator released by occlusion $r = -0.2923,\ p > 0.05$

The pre-occlusion activator level, the post-occlusion activator level and the amount of activator released by occlusion differ significantly in normal controls and in patients with PCH. An inverse correlation was found only between the activator released by occlusion and triglycerides or phospholipids resp. Cholesterol and FFA show only a tendency to be inversely related.

Discussion

The presence of a decreased fibrinolytic activity in the blood of patients with PCH could again be verified in the present study. This diminished activator activity has been found earlier using another assay method, in our laboratory [28]. Similar findings were made by Sweet *et al.* [31] and Blatny *et al.* [6] whereas Nitzberg *et al.* [24] found the dilute whole blood lysis time to be similar in hypertriglyceridemic and hypercholesteremic subjects and controls. Sweet *et al.* [31] were able to calculate a significant inverse correlation between triglycerides and PA (expressed as englobulin lysis time). We could not confirm this last finding although the negative correlation coefficient, found by us too demonstrates a tendency of inverse relationship. Neither could we verify Sweet's report on a tendency for increased FFA levels to be associated with an increase in fibrinolytic activity.

After 10 min of venous occlusion the global activator level as well as the activator released by occlusion differed significantly between normal and hypertriglyceridemic subjects. This deficient reaction on stasis seems to be characteristic for the PCH. The PA released by venous occlusion was found to be inversely correlated with the triglyceride and phospholipid level, indicating that these lipids interfere with the stasis induced activator or its release from the vessel wall. This finding was surprising since we had evidence that triglycerides

or chylomicrons do not inhibit the direct plasminogen activation by urokinase, whether in PCH [30] nor in alimentary hypertriglyceridemia [5 26] nor after infusion of a triglyceride emulsion [10] nor after *in vitro* addition[1] These findings can be explained by means of 2 theories

1 There is a difference in biochemical behaviour between the spontaneous occurring PA in the blood and the PA formed by venous occlusion. The latter may be inhibitable by lipids. The theory of a biochemical difference between the pre- and post-occlusion PA is supported by LATRIDIS *et al.* [19] and by HOLEMANS and ROBERTS [16] Their findings suggest, that the 'post-occlusion lytic activity is predominantly due to an indirect activator of the lysokinase type and intimately related to the Hageman factor The difference in reaction with lipids may also explain the reports on an inhibitory effect of triglycerides on the indirect plasminogen activation by streptokinase [6 15 24] On the other hand this theory may be contradicted by the following a) We had the opportunity to follow up the post-occlusion activator increase in 2 patients with extremely high triglyceride levels (8,950 resp 4 670 mg/100 ml) In these patients, subjected to a strict carbohydrate free regimen, the triglyceride levels were lowered considerably (470 resp 372 mg/100 ml) The expected activator increase did not occur b) we found the streptokinase induced clot lysis time, using the method of PARASKEVAS *et al.* [26] to be similar in hypertriglyceridemic plasma before and after removal of triglycerides by means of ultracentrifugation[1]

2 The decreased fibrinolytic activity following venous occlusion in patients with PCH may be due to an inhibition of the PA release from the vessel wall by triglycerides, possibly in the way proposed by HOWELL [18] and/or reduced contents of PA in the vessel wall in patients with PCH Probably further investigations on the nature and behaviour of the post-occlusion PA will elucidate this problem.

From our findings we can conclude, that patients with PCH exhibit a considerably lower PA level than normal controls and that their reaction on stasis is deficient. It cannot be stated, however to what extent these facts are applicable to the arterial response to thrombogenic influences.

[1] Unpublished data.

Summary

In 29 patients with primary carbohydrate-induced hypertriglyceridemia (triglyceride levels between 200 and 8,950 mg% and clear pre-β-band in the lipoproteinelectrophoresis) and in 24 normal controls (triglyceride levels not over 160 mg%) the plasminogen activator before and after 10 min of venous occlusion was tested. In patients with PCH the activator activity before occlusion and the release of 'post-occlusion' activator is significantly lower than in normal persons. An inverse correlation was found between the activator released by occlusion and triglycerides as well as phospholipids.

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Thalassaemias and Abnormal Haemoglobins in SR Macedonia

A Survey of 2,861 Children

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M Andreeva, G Petkov and C. Lazova

In 1952 Manchev [16] described the first case of thalassaemia major in the SR Macedonia in a boy of Macedonian origin. Since then the number of patients diagnosed as having Cooley's anaemia has steadily increased During a period of 16 years, 50 cases have been admitted to the Department of Pediatrics, Faculty of Medicine, Skopje. They originated from different parts of the country but most of them came from southeastern section of the SR Macedonia. The anomaly has also been found in 3 children of Albanian origin [19] Most of the patients were found to be homozygotes for β-thalassaemia (A_2-thalassaemia) [4 19] a few were double heterozygous for β-thalassaemia and for Hb Lepore, some were homozygous for Hb Lepore [2] while one patient was diagnosed as a Hb H thalassaemia disease [5] The present study was undertaken in order to obtain more information on the frequency and distribution of the various thalassaemia types and other haemoglobinopathies in SR Macedonia.

Material and Methods

Samples of venous blood were collected from 2,861 pparently healthy children aged 7 to 18 years from different parts of the country All children were examined by pediatrician before blood sample was taken in heparinized tube. Another group of 561 cord blood samples was obtained through the services of the Department of Obstetrics, Faculty of Medicine, Skopje.

The quantity of alkali resistant haemoglobin was determined by the method of Betke *et al.* [1] values under 1% were considered to be normal. The Hb A_2 level was determined by starch block lectrophoresis according the technique of Kunkel and Wallenius [14]

with Tris-EDTA (disodium salt)–boric acid buffer pH 9.0 [7] values for Hb A_2 of 3.3% and lower were considered to be normal. Starch gel electrophoresis [22] in Tris-EDTA-boric acid buffer pH 8.9 [11] and in phosphate buffer pH 6.5 [12], was used for the detection and identification of abnormal haemoglobins. Osmotic fragility was determined by suspending erythrocytes in 0.5% NaCl solution at room temperature and visual judgment of the completeness of haemolysis after 6 and 24 h [9].

Results

The 2 861 apparently unrelated children studied came from 32 different places of SR Macedonia 135 exhibited an increased level of Hb A_2, corresponding to an incidence of 4 72% of β-thalassaemia (A_2-thalassaemia) trait carriers. Additional differences were observed, which made it necessary to classify these cases in the following 3 groups (tables I and II)

Group I 64 cases or 32 6 %. Individuals of this group showed elevat

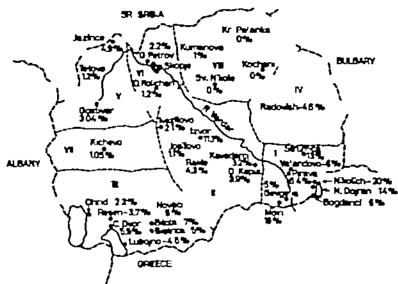


Fig. 1 Geographical distribution of the carriers of β-thalassaemia in SR Macedonia. I = 9.63 (5.0–20.0) % II = 4.69 (1 1 11 1 %; III = 4.65 (2.2–7.0) % IV = 4.63% V = 2.45 (1.2 4.9 % VI = 1.83 (1.2 2.2) % VII = 1.05%; VIII = 0.30 (0–1.0) %.

Table I Incidence of β-thalassaemia and abnormal haemoglobins in SR Macedonia

Place	No. of cases	Group 1 $A_2 > 3.5\%$ $F > 1.0\%$ O.F +	$A_2 > 3.5\%$ $F > 1.0\%$	Group 2 $A_2 > 3.5\%$ O.F +	$A_2 > 3.5\%$	Group 3 $F > 1.0\%$ O.F +	$F > 1.0\%$	O.F +	No. and % of carriers of β-thal. gene from groups 1 and 2	Abnormal Hb
Gevgelia	100	1	1	–	3	1	2	2	5 (5%)	
Bogdanci	100	–	2	1	3	–	3	9	6 (6%)	
N. Dojran	100	2	5	1	6	–	1	1	14 (14%)	
Nikolich	40	3	2	–	3	1	–	1	8 (20%)	
Mola	21	1	1		2		1	1	4 (19%)	
Strumica	100	–	7	–	6	3	3	4	13 (13%)	
Bitola	100	2	2	–	3	–	1	9	7 (7.0%)	
Novaci	60		1		2	–	4	1	3 (5.0%)	
Bistrica	40	–	1	–	1	–	–	–	2 (5.0%)	Hb *D* (1)
Resen	81	2	–	–	1	–	2	2	3 (3.7%)	
C. Dvor	118		3	–	4		1	1	7 (5.9%)	
Radovish	259	1	5	–	6		2	1	12 (4.6%)	
G. Petrov	135	1		–	–	–	2	–	3 (2.2%)	
Izvor	97	1	4		6	–	3	–	11 (11.1%)	
Gostivar	98	1			1	–	3	–	2 (3.04%)	
Jadinci	61	–	1	–	2	–		–	3 (4.9%)	
D Kolicane	83		1		–	–	–	–	1 (1.2%)	
Nailovo	46		1	–	–	–	1	–	1 (2.1%)	
Valandovo	99	3	–	2	1	–	2	–	6 (6.06%)	Hb J (1)
Pirava	63	–	2	–	2		–	1	4 (6.4%)	Hb J (1)
Kavadarci	93		2	–	1	–	1	–	3 (3.2%)	Hb D (1)
Kichevo	95	–	–	–	1		–	–	1 (1.05%)	
Josifovo	87	–	–	–	1	–	–	–	1 (1.1%)	
Kr Palanka	51	–	–	–	–	–	2	–	0	
D. Kapia	77	–	–	–	3	–	1	–	3 (3.9%)	Hb Lepore (1)
Kumanovo	95	–	1	–	–	1	2	–	1 (1.0%)	
R. Prilep	69	1	–	1	1	–	1	–	3 (4.3%)	
Tetovo	86	–	–	–	1	–	1	–	1 (1.2%)	
Kochani	80			–	–	–	2	–	0	
Sv Nikole	54	–	–		–	–	–	–	0	
Ohrid	136	–	1	–	2	–	5	–	3 (2.2%)	
Lobeljan	88	1	1	–	2	–	3	–	4 (4.5%)	Hb Lepore (1)
Total	2,861	20	44	5	08	6	48	33	135 (4.72%)	6 (0.21%)

F — positive osmotic fragility test   O.F — negative osmotic fragility test

Table II. Elevations of Hb A_2 and Hb F and abnormal haemoglobins in SR Macedonia

	A_2 increased F increased	A_2 increased only	F increased only	Abnormal hemoglobins Lepore	J	D
No of cases	64	71	55	2	2	2
Percent of total series (2,861)	2.24	2 48	1.92	0.07	0.07	0.07

ed levels of Hb A_2 and Hb F while some had also an increased osmotic fragility of red cells. In 44 of the 64 subjects, normal osmotic fragility test was found. The mean value of Hb A_2 was 4.93% (range 3 48–6.7%) and of Hb F 2.65% (range 1 0–7.2%)

Group 2 71 cases or 36.2% These individuals showed elevated levels of Hb A_2 (mean value 4 6% range 3 6–5 4%) but normal levels of Hb F (mean value 0.38% range 0–0.96%) Sixty-six cases showed a normal osmotic fragility

Group 3 55 cases or 28.1% These children had elevated levels of Hb F (mean 3 72% range 1.3–8.6 %) but a normal amount of Hb A_2 (mean 2 47% range 1 64–3.24%) Forty-nine cases showed a normal osmotic fragility while 14 cases had values of Hb F below 1.5% (mean value 1.26% range 1 1–1 45%)

Figure 1 shows the geographical distribution of the carriers of β-thalassaemia (A_2-thalassaemia) in the SR Macedonia. The gene for A_2 thalassaemia is distributed through the whole country but it varies from 0 to 20%. According to the incidence, the country has been divided into 8 regions (I) South-eastern part (region of Strumica and Gevgelia) with an incidence of 5–20% (mean value 9 63%) (II) central part (region of T Veles and Kavadarci) with an incidence of 1 1–11 1% (mean value 4 69%) (III) south-western part (region of Bitola and Ohrid) with an incidence of 2.2–7% (mean value 4 65%) (IV) eastern part (region of Radovish) with 4 63% (V) north western part (region of Gostivar and Tetovo) with 1.2–4.9% (mean value 2 45%) (VI) region of Skopje with 1.2–2.2% (mean value 1.83%) (VII) western part (region of Kichevo) with 1 05% (VIII) north eastern part (region of Kumanovo K. Palanka, Kochani) with an incidence of 0–1% (mean value 0.3%)

In the south-eastern and central part of the country the incidence is especially high in certain small communities Nikolich (20%)

Mom (19%) Izvor (11 1%) which is probably caused by isolation and intermarriage. It is of interest that a village in this region (Josifovo) which is known to have been colonized by people from the western part of the country showed low incidence (1 1%) In 2 cities, Strumica and Bitola, where investigations were performed on children living in villages around these cities the incidence of thalassaemia gene was very high (13 and 7% respectively)

Excluding the subjects with elevated levels of Hb A_2 as well as Hb F a total of 6 cases (0.21 ‰) with 3 different abnormal haemoglobins were found (tables I and II) 2 cases with Hb Dβ (3) 2 cases with Hb Jx (20) and 2 cases with Hb Lepore.

Only one case with Hb Bart's (4 7%) was observed among the 561 cord blood samples studied Investigation performed 8 months later showed the presence of Hb Bart's (3.8%) and Hb H (4.2%) these observations made a diagnosis of α-thalassaemia likely The inclusion bodies specific for α thalassaemia were found in one of the parents. In about 80% of the investigated cord blood samples, Hb Bart's was found in a quantity of about 0.5%

Discussion

Although Yugoslavia belongs to the Mediterranean countries where β-thalassaemia is known to be prevalent, no attempt to establish the incidence of this gene has yet been made. Extensive surveys have been carried out in Italy and Greece where these countries have been mapped by the numerous studies of SILVESTRONI and his collaborators [21] and MALAMOS, FESSAS and others [6–8, 10 15]

The present study was undertaken in order to obtain basic information on the incidence of thalassaemias and other haemoglobinopathies in Southern Yugoslavia, i.e. SR Macedonia. It showed a relatively high incidence (4 72%) of β-thalassaemia (A_2-thalassaemia) carriers. In certain restricted areas, the percentage of trait carriers was considerably higher (20%) In 48% of the cases with elevated Hb A_2, the level of Hb F was above 1% which is considered the upper level of normal.

At least 2 genetic subtypes of β-thalassaemia have been recognized, namely the A_2-thalassaemia i.e classical β-thalassaemia, and F thalassaemia [7 18 23] In heterozygotes of the A_2-thalassaemia there

is a distinct elevation of Hb A_2, with normal or slightly elevated values of Hb F (1 to 6%). In F thalassaemia trait carriers Hb A_2 levels are normal while Hb F is always elevated. Our studies indicate that the incidence of β-thalassaemia (A_2-thalassaemia) in the SR Macedonia is 4.72%.

The highest incidence of classical β-thalassaemia is found around the Greece border. On the other side, no Hb S and hereditary persistance of Hb F [8] were found in this study although it is known that these abnormalities are rather common in Greece [6, 8, 10].

Fifty five individuals (1.92%) with levels of Hb F above 1% and normal values for Hb A_2 were found in this study. Since the factors influencing the elevations of Hb F such as familial minor Hb F elevations [13–17] and iron deficiency anemia due to hookworm infestation were not evaluated, this group of cases could not be included at the present time for the evaluation of the incidence of thalassaemia carriers. It seems likely however, that a reasonable number may represent cases of 'F-thalassaemia'.

The osmotic fragility showed no correlation with the levels of Hb A and Hb F. Of the 135 cases with elevated Hb A_2, 110 showed normal osmotic fragility. On the other hand, of the 2,861 screened children, 33 showed increased osmotic fragility without elevation of Hb A_2 and Hb F. It seems that the osmotic fragility of red cells in 0.30% NaCl, as used by other investigators [9] is not an adequate screening test for the evaluation of the incidence of thalassaemia.

Only one case of α-thalassaemia was found in about 560 cases tested. This is likely not the true incidence, since the investigation was performed on a small number of cases and was moreover restricted to the area around Skopje. The incidence of abnormal haemoglobins (Hb Lepore, Hb D_β, Hb Jx) was found to be 0.21%.

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Summary

2,861 healthy children from 32 different places of SR Macedonia, Yugoslavia, and 561 cord blood samples were screened for the presence of thalassaemias and other haemoglobino-

pathies by quantitation of Hb A_2 and Hb F osmotic fragility test, and starch gel electrophoresis. The incidence of β-thalassaemia ("A_2-thalassaemia") was 4.72%. In 48% of the cases with elevated Hb A_2 an increase of Hb F above 1% was found. Fifty-five children (1.92%) showed levels of Hb F above 1% with normal levels of Hb A_2. Most of them are considered to be carriers of 'F-thalassaemia The highest incidence of β-thalassaemia was observed in the south-eastern part of SR Macedonia, i.e. around Greece border (5–20%)

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Recovery of Haemopoiesis after Cyclophosphamide

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In an earlier investigation [13] the degenerative changes taking place in the bone marrow and blood of the guinea pig in the first 6 days after a sublethal dose of cyclophosphamide were studied, using electron microscopic, histological and quantitative methods. A drastic reduction in the cellularity of the marrow affecting all the major cell lines was observed. It further appeared that study of the recovery of haemopoietic tissue following such a profound disturbance of the cellular equilibrium might throw light on underlying haemopoietic mechanisms; it would also provide further information concerning a drug which is used in the treatment of various haematological disorders. The present communication reports the findings of a quantitative study of the bone marrow and blood of the guinea pig over a period of 6–21 days after cyclophosphamide administration.

Material and Method

All the observations were made on healthy male guinea pigs of the Dunkin Hartley strain weighing approximately 400 g at the commencement of the experiment. They were in all respects similar to those used in the earlier investigation [13]. Fifty animals were studied: 25 of these (the test series) each received a single i.p. injection of 350 mg/kg of cyclophosphamide (Endoxana®) and the controls were injected with a corresponding volume of sterile physiological saline. Corresponding test and control experiments were carried out simultaneously.

Control animals showed an average loss of body weight of about 10 g on the first day after injection but thereafter continued to gain weight at the normal rate for these immature animals, namely a little under 10 g per day. Test animals were usually a little off-colour for the first 3 days and lost an average of about 10% of the body weight. The initial body

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weight was re-gained by about the 10th day and thereafter they continued to gain weight at approximately the normal rate, although remaining on average 80 g lighter than the corresponding controls throughout the period of the observations. No other ill-effects were observed.

Five animals per group were studied after 6, 9 12 18 and 21 days. Quantitative studies of bone marrow and blood were carried out by methods essentially similar to those described by YOFFEY [16].

The animals were killed by exsanguination from the common carotid artery. Blood counts were performed by standard methods on arterial blood which was collected during exsanguination and prevented from clotting by sequestrene. A direct method was used to count the number of eosinophils [9].

A plug of marrow was agitated with known volume of autologous serum in small tube containing glass bead, nd uniform cell-suspension was obtained. The volume dilution was calculated. Normal haemocytometric methods were used to count the number of nucleated cells per unit volume of the suspension and the result was converted into total nucleated cells per mm³ of the original bone marrow. Results were obtained for the humeral marrow of both sides of the body and averaged. Smears were prepared from the suspensions, air-dried and stained with MacNeal's tetrachrome stain. Differential counts were made in the usual way and by relating them to the total nucleated cell-counts, bsolute numbers per mm³ of the bone marrow were obtained.

Table I Main nucleated cell groups in bone marrow (in thousands per mm³)

	Total	Myeloid	Erythroid	Lymphoc.	Damaged
6 d Test	528 ± 128	194 ± 54	139 ± 73	67 ± 28	81 ± 36
Control	1,848 ± 89	755 ± 144	472 ± 56	373 ± 112	103 ± 29
t value	18.9	8.5	7.9	5.9	1.2
9 d Test	1,293 ± 62	771 ± 63	111 ± 55	163 ± 29	144 ± 16
Control	1,863 ± 50	752 ± 69	529 ± 78	345 ± 97	103 ± 51
t value	16.0	0.9	6.6	4.0	2.3
12 d Test	1,908 ± 74	1,542 ± 104	133 ± 23	134 ± 61	37 ± 15
Control	1,922 ± 43	766 ± 145	503 ± 119	470 ± 91	88 ± 36
t value	0.4	9.7	6.9	6.8	2.9
18 d Test	1,777 ± 31	761 ± 148	515 ± 83	520 ± 35	99 ± 43
Control	1,842 ± 46	677 ± 43	446 ± 66	502 ± 57	121 ± 37
t value	2.6	1.2	1 4	6.0	0.8
21 d Test	1,897 ± 55	539 ± 124	608 ± 81	491 ± 74	132 ± 32
Control	1,873 ± 61	632 ± 108	511 ± 99	489 ± 33	114 ± 26
t value	0.6	1.5	1.7	0.1	1.0

± standard deviation.

The values are those of the standard error test for small samples. They were calculated following normal statistical procedures. Here, t value exceeding 2.3 indicates significance at the 0.05 level of probability and one exceeding 3.4 indicates significance at the 0.01 level.

The absolute counts of megakaryocytes, macrophages, plasma cells, monocytes and unidentified cells have not been separately itemized, although they are included in the total nucleated count.

Results

Bone marrow. A summary of the data for the total nucleated cells and the main cell groups is given in table I. The more detailed findings for the myeloid, erythroid and lymphocyte subgroups are summarized in tables II and III. Unless otherwise stated, attention will only be drawn to differences between test and control groups which were significant at the 0.01 level of probability. Table I shows that the cellularity of the bone marrow was decreased at 6 and 9 days after cyclophosphamide. Thereafter there were no striking differences in cellularity between test and control groups. Myeloid cells (granulocyte precursors) were greatly reduced in numbers at 6 days after cyclophosphamide (table I) the difference being largely the result of a drastic reduction in the numbers of band and segmented neutrophils (table II). At 9 days, although no difference between the total numbers of myeloid cells was detected (table I) there were increased numbers of neutrophil promyelocytes, myelocytes, metamyelocytes in the test

Table II. Myeloid cell subgroups (in thousands per mm^3)

	Myeloblasts	Promyel. + myel.	Metamyel. neutro.	Band + segm. neutro.	Other neutro.	Eosino.
6 d Test	17± 9	63±30	61±31	10± 2	30±19	3± 4
Control	27±17	76±53	106±20	400±103	63±30	68±13
t value	1.0	0.4	2.8	8.0	2.1	10.4
9 d Test	32±22	247±72	267±32	152±59	67±18	3± 3
Control	25±12	93±47	126±19	392±72	35± 7	67±32
t value	0.6	4.0	8.4	5.8	3.7	4.5
12 d Test	13± 8	61±17	435±78	957±97	70±19	3± 3
Control	19± 4	71±32	165±43	400±117	44±20	56±40
t value	1.6	0.6	7.3	8.2	2.1	2.4
18 d Test	16± 9	37±15	79±19	522±142	32±21	53±23
Control	23± 5	48±12	99±27	341±56	66±26	82±23
t value	1.4	1.2	1.3	2.6	2.2	1.8
21 d Test	30±11	35±13	64±21	274±83	66±43	72±46
Control	21± 3	48±14	110±37	297±39	63±28	93±35
t value	1.8	1.5	2.3	0.5	0.1	0.9

See footnotes to table I. Unidentified, damaged and degenerating neutrophils are included in the totals labelled 'Other Neutro'. The absolute counts of basophils have been omitted.

Table III. Erythroblasts and lymphocyte subgroups (in thousands per mm³)

	Pro-eryth.	Baso. erythr	Poly + ortho. eryth.	Small lymph	Transi-tionals
6 d Test	4± 2	12±14	119±39	38±22	29±10
Control	14±11	31±14	421±53	241±103	132±23
t value	2.0	2.0	8.5	4.3	8.4
9 d Test	6± 6	14±11	88±46	26± 9	138±33
Control	17±12	43±13	439±[illegible]	201±89	144±25
t value	1.7	3.6	11.2	4.3	1.4
12 d Test	9± 3	13±12	102±20	22±15	111±50
Control	23± 5	43±17	417±118	291±70	179±36
t value	5.3	4.9	6.0	8.6	2.5
18 d Test	28± 8	37±12	424±63	141±25	179±24
Control	24± 6	28± 9	368±65	305±67	197±21
t value	0.8	1.4	1.3	5.0	1.3
21 d Test	23± 4	43± 5	520±74	300±70	191±25
Control	20±13	33± 8	438±93	319±42	170±17
t value	0.6	2.3	1.5	0.5	1.7

See footnotes to table I. The absolute counts of extruded nuclei of erythroblasts have been omitted. 'Transitional' includes medium lymphocytes and transitional cells [16].

group while the band and segmented form neutrophils were still reduced in numbers (table II) All 4 neutrophil compartments in table II were strikingly higher at 9 days than at 6 days, the t values being promyelocytes and myelocytes, 5.2 metamyelocytes, 10.2 band and segmented forms, 5.3 other neutrophils, 3.1 At 12 days after cyclophosphamide there were more than twice the normal number of myeloid cells, the neutrophil metamyelocytes and the band and segmented forms showing corresponding increases in their absolute numbers. The promyelocytes and myelocytes, on the other hand, were within normal range. At 18 and 21 days, there were no striking differences between the myeloid series of the test and control groups. *It is, however worth noting that at 18 days, the band and segmented* neutrophil compartment of the test group was greater than that of the corresponding control group the difference reaching significance at the 0 05 but not at the 0 01 level.

The total erythroid cells were markedly reduced in number at 6, 9 and 12 days after cyclophosphamide (table I) the differences being

largely the results of corresponding differences in the polychromatic and orthochromatic erythroblasts (table III) The numbers of pro-erythroblasts and basophilic erythroblasts were also smaller after these intervals, but the differences at the 6th day (and in the case of the proerythroblasts at the 9th day) did not reach the conventional level of probability No marked differences in the erythroid cells were noted at the 18th and 21st day

The total lymphocytes (table I) were reduced in numbers at all the periods of observation except the 21st day The changes in small lymphocytes (table III) followed an identical pattern. There was on average, a more than four-fold increase in the number of transitional cells and medium lymphocytes between the 6th and 9th days. The average figures for transitional cells alone showed a similar increase from 15 000 to 52 000 per mm³ the t value for the difference being 3.6 Thereafter no marked differences were found between test and control groups with regard to these cells.

Blood. The total white cell counts of the blood (table IV) were reduced on average to approximately 20% of control values on the

Table IV White blood cells per mm³ and spleen weights in g

	Total cells × 10	Neutro. × 10	Eosino.	Mono. × 10	Spleen weight, g
6 d Test	120±30	7± 5	7± 4	106±28	0.34±0.08
Control	597±88	284±74	17± 7	293±50	0.66±0.14
t value	11.5	8.4	3.0	7.3	4.3
9 d Test	293±86	79±52	2± 2	202±37	0.55±0.04
Control	547±88	220±69	17± 7	309±64	0.64±0.09
t value	6.4	3.7	4.7	3.5	1.9
12 d Test	1,508±707	990±790	11± 8	354±223	1.23±0.19
Control	627±145	286±123	30±11	310±58	0.65±0.04
t value	2.7	1.9	3.8	0.3	6.5
18 d Test	501±252	280±155	30±29	186±153	0.92±0.17
Control	474±106	140±73	40±13	293±157	0.64±0.07
t value	0.6	1.7	2.3	1.6	3.3
21 d Test	421±53	210±53	29± 7	181±47	0.73±0.13
Control	413±72	114±48	37±17	231±35	0.74±0.05
t value	0.2	1.9	0.9	2.2	0.03

See footnotes to table I. Mononuclears include lymphocytes, monocytes and blast cells.

6th day and a decrease in numbers was shown in all the cell types counted. At 9 days, similar differences between test and control values were present, although they were less marked. At 12 days there were, on average, over twice as many white cells in the blood of the test animals as in the controls, the difference being significant at 0.05 level; thereafter no striking differences were observed. The eosinophil counts of the cyclophosphamide-injected animals were markedly below control levels in the 6, 9 and 12 day observations.

Spleen weight. 6 days after cyclophosphamide the spleen weight was on average only about half that of the control value, whereas by the 12th day it was almost double the control weight. By the 21st day no significant difference could be detected.

Other observations. In the marrow smears of the test series at 6, 9 and 12 days after cyclophosphamide, one encountered cells which had the size and cytoplasmic characteristics of immature neutrophil granulocytes, while the nucleus showed a varying number of separate segments, often more than 10. The average counts of these 'segmented metamyelocytes' per mm³ of bone marrow were 8 000, 7 400 and 20 600 at the 6th, 9th and 12th day respectively.

Discussion

The administration of a sublethal dose of cyclophosphamide brings about a striking reduction in the marrow cellularity affecting all the major cell-lines [7, 13]. The present results indicate that the patterns of regeneration are different for each of these cell-lines; they will therefore be considered separately. It is perhaps necessary to add that recovery has only been studied over a period of 21 days and that long term effects such as occur after irradiation [11, 12] have not been excluded. Furthermore, the changes in the spleen weight (table IV) and particularly the striking increase in weight at 12 days after cyclophosphamide serve to emphasize that the patterns of recovery seen in the bone marrow need to be considered in relation to changes in the rest of the lympho-myeloid complex.

Granulocytes

The recovery of the neutrophil granulocytes appeared to follow an

orderly sequence, the early forms recovering before the later As noted in the previous investigation, the early granulocyte precursors (myeloblasts, neutrophil promyelocytes and myelocytes) showed evidence of an increase in their numbers between the 3rd and 6th days after cyclophosphamide. By the 6th day their numbers were no longer significantly different from those of the controls (table II) By the 9th day commencing recovery in the later neutrophil compartments was evident, in that their numbers were significantly larger than at 6 days. The blood neutrophils showed similar evidence of commencing recovery at 9 days. This time lag between the detection of commencing recovery in the early neutrophil precursors on the one hand and the later forms and blood neutrophils on the other presumably reflects the time needed for cell-maturation in these circumstances. A similar time-lag was noted by HARRIS [4] and HULSE [10] in the neutrophil series of animals recovering from exposure to irradiation. A similar time lag was also observed in the degeneration pattern of the neutrophil series after the administration of cyclophosphamide, which appeared mainly to affect the mitotically active forms [13]

One of the features of the pattern of recovery was a marked over shooting' of the normal neutrophil values. This overshooting appeared to follow a similar sequence, in that it was most marked in the neutrophil promyelocytes and myelocytes at 9 days, in the metamyelocytes at 9 and 12 days, and in the band and segmented forms at 12 days. This overshooting is in agreement with the observation of HOFF [7] who obtained values above normal 8–12 days after the injection of cyclophosphamide in the rat. A similar overshooting was also observed by ELSON *et al.* [2] 10 days after the injection of chlorambucil in the rat. The interpretation of this striking increase in neutrophils presents some difficulties. There was no external evidence of infection and at this time, the animals were eating well and gaining weight. It seems more likely that the overshooting is related to a disturbance of the complex mechanisms controlling the production and release of these granulocytes [1 15] One possible factor in relation to this was the presence up to the 12th day of the abnormal neutrophils which have been termed segmented metamyelocytes These large cells which show an unusual degree of segmentation in the nucleus, have an abnormally high content of DNA [13]

By the 21st day the granulocytes had completely regained control levels. No evidence was seen of an abortive rise and secondary fall in

neutrophil numbers, such as has been observed following irradiation in both guinea pig and rat [4 10] Host [7] in experiments designed to compare the effects of irradiation and cyclophosphamide in the rat, confirmed the presence of a secondary fall in neutrophil numbers following irradiation but did not observe any secondary fall after cyclophosphamide. These observations suggest that the pattern of recovery following cyclophosphamide is different from that following irradiation [7 8] although the possibility that there might be a secondary fall in neutrophil numbers after the 21st day has not been excluded. Dosage differences might also be of importance.

Red Cell Precursors

The most striking repopulation of the erythroid cells in the bone marrow took place between the 12th and 18th day The average number of erythroid cells at 18 days after cyclophosphamide being almost 3 times the corresponding number at 12 days (table I) and each of the subgroups had reached control levels by the 18th day (table III) While major recovery seemed to have taken place during this period, slight recovery occurred prior to this. As already noted [13] all the 3 groups showed a small but significant increase in their numbers between the 3rd and 6th day after cyclophosphamide and this recovery was maintained with minor fluctuations until the 12th day

There was no evidence of 'overshooting' of erythroid numbers, although this could conceivably have occurred between, for example, the 12th and 18th day The absence of overshooting would be consistent with the findings of Host [7] following cyclophosphamide treatment in the rat. Abnormally high erythroid counts have however been observed during the recovery from irradiation [3 11]

Lymphocytes

There was a difference between the patterns of recovery of the small lymphocytes on the one hand and the transitional cells and medium lymphocytes on the other Up to the 12th day the results gave no indication of commencing regeneration in the small lymphocyte population. By the 18th day however recovery had commenced and by the 21st day control levels had been regained. On the other hand

the results indicated that restoration of transitional cells and medium lymphocytes took place much earlier, the major recovery occurring between the 6th and 9th days (table III). There was some evidence suggesting a secondary fall at the 12th day, but this must be treated with reserve in view of differences between different control groups.

The pattern of recovery of the small lymphocytes of the bone marrow was similar to that found by HOTT [7]. He reported that after the 10th day the lymphocyte-like cells rose only to subnormal or normal values, whereas following irradiation, these cells rose to levels considerably above normal. The latter observation confirmed the findings of HARRIS [3, 5] in guinea pigs. The rise in marrow lymphocytes has been shown to depend on the dose of irradiation [12]. By means of studies of DNA-synthesis HARRIS *et al.* [6] also showed that a considerable proportion of the small lymphocytes in the irradiated marrow were newly formed.

The early recovery of the transitional cells may be particularly significant. These cells are known to be actively proliferating [6, 17] and there is evidence suggesting that they may possibly function as stem cells [14].

The factors underlying these differences in recovery pattern are not always clear. One factor has already been mentioned, namely that earlier appearing cells may be precursors of later-appearing forms. The speed of recovery might also reflect the importance of a cell-line to the body's defence mechanisms in that particular set of circumstances. One could also speculate that the alkylating agent might have affected some of the surviving stem cells in such a way that one particular type of differentiation could more readily take place than another. Further work with the object of investigating the fundamental significance of the varying patterns observed here might be rewarding.

Acknowledgements

We thank Miss G. HARDEVO for technical help and Miss D. DOLAN for secretarial assistance.

Summary

Quantitative studies of bone marrow and blood were carried out in guinea pigs at intervals of 6–21 days after a single sublethal dose of cyclophosphamide. The pattern by which the bone marrow recovered from the initial depopulation differed for each of the major cell-

lines. One of the features of the neutrophil granulocyte recovery was that marked overshooting of control values was observed at 9–12 days changes in the early forms were in general detected few days before those in the later forms. Segmented metamyelocytes were prominent in the marrow until the 12th day The main recovery of the erythroid and small lymphocyte populations occurred in the third week after cyclophosphamide whilst that of the transitional cells and medium lymphocytes took place much earlier I seems likely that these differences in recovery pattern reflect the complex interplay of variety of factors.

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Thrombotic Thrombocytopenic Purpura

Report of a Case Treated with Splenectomy and Steroids

J. R. Scharoff, N. Serlin and M. A. Atamer

Since the original description of thrombotic thrombocytopenic purpura by Moschcowitz in 1925 [1] fewer than 300 cases have been reported in the literature [2]. The most commonly applied name thrombotic thrombocytopenic purpura (TTP) is an incomplete term which fails to include hemolytic anemia. Recently there has been a tendency to re-classify this disease under the term of micro-angiopathic hemolytic anemia which includes entities like hemolytic uremic syndrome, malignant hypertension, acute necrotizing glomerulonephritis associated with anemia and often with thrombocytopenia [3, 4].

The disease was formerly considered by most to be uniformly fatal with no effective therapy available [5, 6, 7] despite vigorous trials by several investigators with antibiotics, anticoagulants (particularly heparin), steroids alone, and splenectomy alone. However over the past 10 years, cases have been reported with better results: one patient survived 8 years after treatment with corticosteroids and splenectomy [8].

The following reported case was successfully treated with corticosteroids and splenectomy. It illustrates the importance of studying the renal status even though the patient may show no more hematological and neurological involvement.

Case Report

A 21-year-old white female was admitted to the hospital on April 22, 1965 with chief complaint of weakness and dizziness. The history, given by the patient's husband, included headache, vertigo and paraesthesia of the right hand beginning 18 days *post partum*. One day later she appeared drowsy; she was admitted to the hospital 21 days *post partum*. Her past history was unremarkable. She had 2 normal pregnancies. There was no history of allergies or idiosyncrasies to drugs.

Physical examination. An acutely ill, well-nourished, well-developed jaundiced female, with purpuric spots over her entire body BP 120/80 mm Hg the pulse rate was 78/min liver and spleen were not palpable.

The patient was awake but somewhat disoriented. She was at times unable to comprehend questions and commands. There was some apparent weakness of the left lower extremity Funduscopic examination showed some hemorrhagic areas and engorgement of retinal vessels in the left fundus.

There was severe anemia *Hematocrit 12%, hemoglobin 2.6 g%. Leukocytes 9,900/* mm³ with segmented neutrophiles 72%, juveniles 8%, eosinophiles 1%, lymphocytes 18%, monocytes 1%. Basophilic stippled and fragmented erythrocytes with polychromasia were noted on smear. Ten nucleated red cells were found per 100 WBC. Platelets 27,340 mm³ reticulocytes 19.5%. Sternal bone marrow: normoblastic hyperplasia and increased numbers of megakaryocytes. Urine 1015 sp g 8–10 erythrocytes, 2 3 white cells per high power 2 proteinuria. Glucose 122 mg%; BUN 22 mg%; bilirubin total 3 7 mg% (direct 2 mg%, indirect 1.7 mg%); SGOT 58 units; SGPT 17 units SLDH 6,15 units; fibrinogen 165 mg% bleeding time 3 min; clotting time 4 min 37 sec. Direct and indirect Coombs tests were negative. Clot retraction test showed no retraction after 3 h. Sedimentation rate 47 mm/h [Westergren]

After transfusion with 1,000 ml of whole blood on the first hospital day the patient was started on steroid therapy Prednisone 120 mg/day was given for 4 days, then increased to

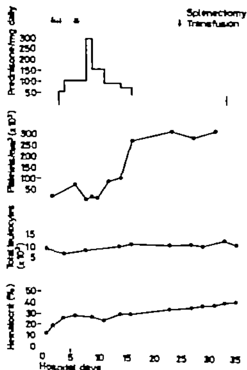


Fig 1 Hematological course

300 mg for 2 days. Meanwhile she was again transfused with 4 units of whole blood and 2 units of packed cells. The hematological course is shown in figure 1

Biopsy of muscle skin and lymph node were performed (see below)

Patient's clinical course continued to worsen in spite of high doses of steroids. She became extremely disoriented on the 6th hospital day showing no signs of comprehension, very

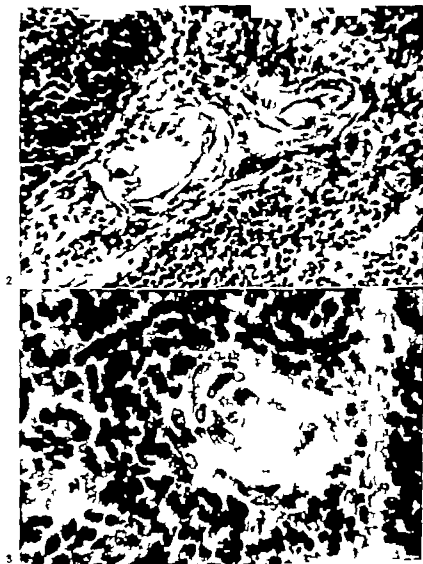


Fig 2. Microscopic section of lymphnode Thrombi nd thickening of the small blood essels.

Fig 3. Microscopic section of the spleen Occlusion of small vessels with reduplication of intima.

irritable. She was bleeding profusely from the sites of biopsy On the 8th hospital day her platelet count was 13,250/mm³ Urine 4+ proteinuria. BUN 42 mg%.

One day later splenectomy was performed. At the time of surgery the patient was transfused with one unit of whole blood and one unit of platelet concentrate.

Microscopic examination of the spleen, lymph nodes, skin and muscle revealed several large and small blood vessels with marked thickening of their walls. Fresh, hyaline thrombi were seen in the latter There was reduplication of the lamina in some areas. The histological findings in these tissues were compatible with thrombotic thrombocytopenic purpura (fig. 2 and 3).

The patient began to improve post-operatively She was placed on 150 mg of prednisone daily then 50 mg daily and continued to improve gradually Hematocrit 30% hemoglobin 8.6 g%; platelets 104,000/mm³ Urine continued to show 4+ proteinuria BUN 41 mg%. Ten days post-operatively hematocrit was 34% hemoglobin 11.0 g%; total bilirubin 2.2 mg% direct bilirubin 1.4 mg% and platelets 297,000/mm³ Immunoelectrophoresis showed no abnormalities.

Patient was discharged 34 days after admission. At this time her mental status was improved, although there was some residual difficulty in comprehending She had some left-sided weakness, with increased reflexes, and was receiving 22.5 mg of prednisone/day This dose was decreased gradually to 2.5 mg every third day until she was completely weaned off steroids.

The patient has been followed for almost 3 years since her discharge without evidence of recurrence of hematological or neurological abnormalities, but the proteinuria continued.

Two years after the diagnosis of TTP had been made a renal biopsy was done. It revealed slight to moderate hypercellular glomeruli with some thickening of the basement membranes, BOWMAN's capsule and some tubules, patchy interstitial round cell infiltration and focal tubular atrophy and degeneration (on H and E and PAS staining) No thrombotic lesions were observed. The histologic pattern was interpreted as being compatible with some mild, diffuse proliferative and membranous glomerulonephritis (fig 4) Direct staining of the tissue obtained with renal biopsy with fluorescein conjugated anti-immunoglobulin serum revealed a small amount of immunoglobulins scattered non-specifically throughout the specimen. At that time the laboratory results were as follows hemoglobin 13 g% hematocrit 40%. WBC 16,100 with segmented neutrophiles 77% lymphocytes 22% and eosinophiles 1%. Platelet count 318,000/mm³ Serum electrophoresis revealed normal distribution of proteins. Complement (C'_3) 125 mg% (normal 145 ± 22 mg%) BUN 15 mg%; creatinine 0.8 mg%; sodium 135 mEq/l; potassium 3.7 mEq/l chlorides 104 mEq/l; CO (CP) 21%; total cholesterol 260 mg%. PSP test showed 27.5% excretion in 30 min. A spot urine test showed 4+ proteinuria occasional granular casts, 6–10 WBC/HPF On overnight concentration, she was able to concentrate to 676 mOs per kg VDRL, LE, rheumatoid factor anti-nuclear antibody tests were all negative. IVP revealed no abnormality

Discussion

Thrombotic thrombocytopenic purpura should be suspected in any patient with fever thrombocytopenia, hemolytic anemia, and transient bizarre neurological manifestations. The characteristic constellation of laboratory data reinforces the clinical diagnosis, and the histological confirmation may be proven at splenectomy or by lymphnode biopsy [9] Although the course is usually fulminating with a fatal outcome,

Renal function tests and biopsy were performed by Renal Laboratory Grønlands Hospital



Fig 4 Microscopic section of renal biopsy Slightly hypercellular glomeruli with some thickening of the basement membranes, BOWMAN's capsule, and some tubules. Pat by interstitial round cell infiltration; focal tubular atrophy and degeneration. H and E staining.

the chronic form of TTP should be suspected in any patient suffering repeated attacks of an acquired hemolytic anemia and/or purpura.

In the past, therapy in TTP has offered only slight hope of averting early deterioration and death Transfusion is notoriously futile the early demise of both red cells and platelets being the usual result. Anticoagulants, heparin in particular offer no aid in combating the occlusive process and may exacerbate hemorrhagic processes. Despite the febrile nature of the acute disease, antimicrobials have proved to be of no value in the treatment of TTP

Recently a successful management with high doses of adrenocortical steroid and dextran on one patient was reported but further trial is obviously indicated [10] Steroids and ACTH alone have produced varying results, but in general have been disappointing

In reviewing the literature of the past 10 years, we have found 9 cases successfully treated with steroids and splenectomy [8 11 12, 13, 14] Of these 5 were females from 27 to 40 years old One of the female cases showed a 5 year remission without any evidence of recurrence [11] Another of the females reported, showed an LE phenomenon

7 months after splenectomy [13] Of the 4 male patients from 21 to 55 years old one had an 8 year survival without relapse [8]

In our case evidence is presented for the continuation of renal involvement despite absence of hematological and neurological manifestations. How often renal disease in patients recovered from TTP after treatment with steroids and splenectomy occurs is unknown, but deserves much more attention than previously given.

Summary

A case of thrombotic thrombocytopenic purpura in 21-year-old female is described. After trial with high doses of steroids, she was splenectomized and made an uneventful recovery. She was followed up for 3 years without evidence of hematological or neurological manifestations of her disease. I the literature 9 similarly treated cases are reported, which all hav recovered. This suggests that this mode of therapy provides more hopeful prognosis in this relatively rare disease

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Refractory Anemia with Leukemic Transformation and Chromosomal Change

A Case Report

Simone Silberman and Eva Krmpotic

We are reporting a case of refractory sideroblastic anemia terminating in an acute myeloblastic leukemia with chromosomal changes in the leukemic end-stage.

Case Report

A 74-year-old man came first to our attention in 1962 because of anemia. Prior to that period, he had a normal hemogram. Except for exertional dyspnea, back pain and urinary frequency the history was not contributory to the diagnosis. There was no history of drug exposure. In 1955 the patient had an active duodenal ulcer treated by diet alone.

He was pale without jaundice. Lymph nodes were not enlarged. The liver was palpable 2 cm below the costal margin and rectal examination revealed a grade II prostatic enlargement.

Initial laboratory examination showed the following values: hemoglobin 9 g%, hematocrit 26%, red blood cells 2.56 $\times 10^6$/mm, MCV 102 μm^3, MCHC 35%, reticulocytes 0.4%, platelets 347,000/mm^3, white blood cells 5,300/mm^3 with a normal differential count and 1 2 nucleated red cells. The bone marrow was hyperplastic with an M:E ratio of 1:1 (fig. 1). Many ringed sideroblasts and increased iron stores were seen (fig. 2 and 3). No megaloblastoid features were present. There was evidence of granulocytic hyperplasia without suggestion of replacement of the marrow by a predominately erythroblastic proliferation. The serum iron was 92 μg/100 ml and the iron binding capacity was 359 μg/100 ml. Serum protein, hemoglobin electrophoresis and fecal urobilinogen were normal. Serum folate and vitamin B_{12} levels showed no abnormalities. Liver function studies were also normal.

When the patient was seen again in 1964 the spleen was felt 2 cm below the costal margin. There was little change in the hemogram and an examination of the bone marrow revealed essentially the same findings. Peripheral smears stained with PAS showed no glycogen or other polysaccharides within normoblasts. The leukocyte alkaline phosphatase was within normal limits. In 1965, the patient underwent a retropubic prostatectomy for adenocarcinoma of the prostate and received two units of packed cell. Additional 6 units of blood were given after bilateral orchidectomy.

Fig 1 Bone marrow film on admission showing erythroid hyperplasia (May-Grünwald-Giemsa stain) (× 800)



Fig 2. Ringed sideroblasts from bone marrow smear (Prussian blue reaction) (× 800).

Fig 3. Electron micrograph of mitochondria loaded with iron (× 44,000).

His final admission was in January 1967. He presented with severe anemia, cardiomegaly, crepitant rales over the chest and 3 + pitting edema. His hemoglobin was 5 g%

Fig 4 Marrow film before death showing presence of blast cells (May-Grünwald-Giemsa stain) (× 800)

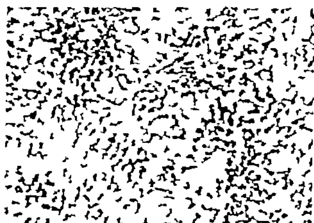


Fig 5. Histologic section of liver with extensive hemosiderosis (Prussian blue reaction) (× 63)

hematocrit 15 %, red blood cells 1.46 × 10^6/mm³, white blood cells 3,400/mm³ with 39% polys, 8% stabs, and numerous normoblasts in the peripheral smear. Serum bilirubin was

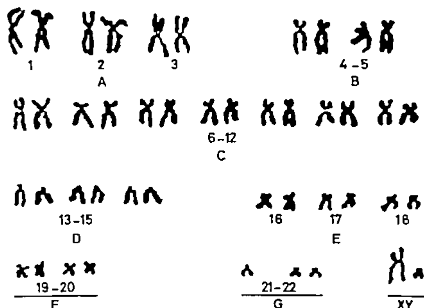


Fig 6. Karyotype of the patient. Note monosomy G21

2 mg% total with 1.2 mg% direct. The bone marrow showed 25% undifferentiated blasts (fig 4). The clinical picture was rapidly deteriorating and one week after admission the patient went into shock and expired.

At autopsy the liver weighed 2,450 g, was firm, dusky brown with finely granular surface. Microscopic examination revealed large septal fibrous bands connecting portal triads with central veins, with formation of regenerative liver nodules. Large amounts of hemosiderin granules were found in the hepatic cells mainly in the lobular periphery (fig 5). The pancreas showed diffuse fibrosis with abundant iron pigment deposition. The spleen weighed 450 g and showed moderate hemosiderin accumulation. Liver, lymph nodes, adrenal glands, heart, thyroid, and collecting tubes of the kidneys contained heavy iron deposits. There was nowhere evidence of visceral infiltration by abnormal erythroid or myeloid cells. The cause of death was an acute apical myocardial infarction.

Chromosome studies were done on the bone marrow in the leukemic stage. A hypodiploid chromosome complement appearing to be monosomy G was observed in 50% of 62 metaphases examined (fig 6). The missing chromosome was small acrocentric chromosome believed to belong in the G group since chromosome with typical Y morphology was present in the hypodiploid metaphase. No structural abnormality of the 19–20 chromosomes was observed in the hypodiploid as well as the normal metaphases from direct bone marrow preparations.

Discussion

Chromosomal abnormalities in idiopathic sideroblastic anemia have been previously reported. These changes included abnormalities in

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Varia

IVth International Symposium on Comparative Leukemia Research

The IVth International Symposium on Comparative Leukemia Research will be held in the USA at the Cherry Hill Inn, Cherry Hill, N.J. from September 21–25, 1969. The Symposium will take place under the auspices of the World Committee for Comparative Leukemia Research and the World Health Organization. During the 4-day meeting, 7 scientific sessions will be held covering etiology, epidemiology, pathogenesis, detection, control and prevention of leukemia in animals and in man.

A $45.00-registration fee will be charged for all active members of the Symposium. Accompanying members' family will be required to pay $30.00-registration fee per person. All participants and attendees will be accommodated at the Cherry Hill Inn at a special low rate for the Symposium period: single occupancy $98.00; double occupancy $77.00 per person. This package plan will include lodging from Sunday night through Thursday night, all breakfasts, coffees, luncheons, one dinner, two receptions, and the final banquet.

Further information concerning the Symposium can be obtained by writing to either Dr. Ray M. Dutcher, General Chairman, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, Pa. 19348 (USA) or to Dr. H.-J. Bendixen, Secretary of the World Committee for Comparative Leukemia Research, Statens Vet. Serumlab., Howitzvej 13, Copenhagen F, Denmark.

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(Head Prof. E. Storti)

Synovectomy, a New Approach to Haemophilic Arthropathy

E. Storti, A. Traldi, E. Tosatti and P. G. Davoli

The earliest, most frequent and most disabling manifestation of haemophilia A and B is acute haemarthrosis. The knee is most frequently affected [1 2, 3 4 5] followed by the elbow and the tibiotarsal joint. Treatment of single or recurring haemophilic haemarthrosis is today still mainly conservative. The serious complications of haemarthrosis (stiffness, deformity ankylosis) have been treated by conservative orthopaedic procedures or surgery. This last method was rarely employed and its scope was arthroadesis or corrective osteotomy. In general, surgical treatment has yielded poor functional results and has always been complicated by serious haemostatic difficulties [3 12, 13]

It may be that in the near future prognosis of haemophilic arthropathy will improve, especially if, from the onset of the bleeding episode, plasma or plasma fractions, rich in factor VIII, will be infused. However it is difficult to foresee the way in which it is possible to prevent further recurrences of haemarthrosis. For the latter reason and in an attempt to obtain a more permanent improvement, we have introduced the operation of synovectomy, the rationale of which is based on the following facts.

In haemophilic articulations, especially the knee, affected by repeated haemarthrosis, after the second or third bleeding episode one may observe local hyperthermy and an increase in the thickness of joint tissues: in other words an infiltrative process is taking place.

These phenomena are also noted in the period following the haemorrhage; even if there is no evidence of fluid accumulation in the joint. Much later in most cases, stiffness and radiologic alterations of both cartilage and bone develop. These radiological and clinical signs (the histological changes of the haemophilic articulation are little known) [6, 7 8, 9]

Table I

Case	Age years	Weight kg	Concentr of AHG or PTC %	Synovectomy articulation	Date	Haemostatic fresh frozen plasma and fresh blood, ml
1	5	17	AHG <1	right knee	13. 4 66	plasma 10,330 blood 1,000
2	8	26.5	PTC <1	right knee	3. 10. 66	plasma 16,330 blood 2,500
3	12	34.5	AHG <1	left knee	20. 1 67	plasma 9,900
4	9	25	PTC =1	left knee	23. 2. 67	plasma 7,950
4	10	27	PTC =1	right knee	17 3. 68	plasma 2,300
5	15	58	AHG <1	right knee	20. 3. 67	plasma 6,000
6	13	55	AHG <1	left knee	20. 3. 67	plasma 8,000
7	7	43	AHG <1	right knee	10. 4. 67	plasma 2,100
8	17	55	PTC =1	right knee	19 6. 67	plasma 3,300
9	14	30	AHG <1	right knee left knee	19 6. 67	plasma 7,650
10	7	24	PTC <1	right knee	26. 6. 67	plasma 2,400
11	13	48	AHG <1	right knee	28. 6. 67	plasma 3,400
12	7	23	AHG <1	tibio-tarsal right joint	13. 3. 68	plasma 1,200
13	6	19	AHG <1	left knee	28. 10. 68	plasma 1,800
14	16	56	AHG <1	left knee	11 11 68	plasma 2,400

led us to believe that the synovial membrane undergoes a conspicuous process of hypertrophy after repeated haemorrhages.

We believe that in recurring haemarthrosis, there are alterations analogous to those noted in other chronic arthropathic disorders, namely haemarthrosis of angiomatous synovial disease and recurring hydrarthrosis of the various forms of synovitis.

On the basis of the bove suppositions we injected corticosteroids into the knee joint of number of haemophiliacs to reduce local tissue reaction. Reduction of these reaction occurs in certain percentage of cases of recurring hydrarthrosis. The result of cortisone therapy was favourable but not lasting. These facts have been reported by other authors [10] Since cases of angiomatous synovial disease and certain forms of hyperthrophic synovitis are treated with synovectomy when corticosteroid treatment fails [11] we attempted synovectomy in florid haemophilic haemarthrosis of the knee.

For the sake of brevity the data concerning our cases are reported in tables I II and III We have performed 16 synovectomies (15 of the knee, 1 of tibiotarsal articulation) in 14 patients. Synovectomy was carried out on one knee in 11 patients and on both knees in 2 patients. In one of these last patients (case 4) the bilateral synovectomy was accomplished in 2 successive operations, the other (case 9) in one single

Table I (continued)

Therapy antifibrinol. comp. parotid inhib. *(in toto)* UIC	EACA g	Intra- and postoperative haemorrhages at site of operation	1st mobilization days after operation
–	–	continuous for 30 days	50
–	–	continuous for 28 days	40
50,000	242	none	15
100,000	220	none	12
600,000	450	none	12
650,000	656	none	12
525,000	678	none	14
625,000	172	none	8
300,000	562	none	12
right 150,000	1,258	modest bilateral,	right 20
left 150,000		on 13th day	left 20
200,000	738	modest, on 10th day	15
300,000	612	none	12
300,000	144	none	8
300,000	372	none	11
400,000	592	none	16

operation. The first synovectomy of the knee was carried out in April 1966 for the other 11 operations, more than two years have elapsed.

Serious difficulties caused by insufficient haemostasis were encountered in the first two cases, whereas in the remaining cases haemostasis was kept under control by addition of antifibrinolytic compounds to the use of plasma.

Results pertaining to articular function are summarized in table II. Table III compares the number of haemorrhages in sites other than that of the synovectomy in the year preceding synovectomy and the total number of haemorrhages in the year following the operation, and the amount of fresh plasma and fresh whole blood or plasma fractions administered during these 2 different periods.

Surgical Procedure

For synovectomy of the knee, the incision is arched, antero-lateral, para-rotular and extends from the superior pole of the rotula to 1 cm below the inferior pole. This incision,

Table II

Case	Synovectomy articulation	Date	Haemarthrosis in operated knee joint: last year before operation	Haemarthrosis in operated knee joint: after operation	Joint function: before operation	Joint function: after 1 year or more from operation
1	right knee	13. 4 66	continuous bleeding	none	flexed t 40°	normal flex. and ext.
2	right knee	3. 10. 66	10	none	flexed at 30°	normal ext., flex. 30°
3	left knee	20. 1 67	1 lasting 20 days	none	normal ext., flex. 60°	normal flex. and ext.
4	left knee	23. ∠. 67	continuous bleeding	none	flexed at 50°	normal flex. and ext.
4	right knee	17 3. 68	2	none	flexed at 20°	normal flex. and ext.
5	right knee	20. 3. 67	continuous bleeding	none	normal ext., flex. 75°	normal flex. and ext.
6	left knee	20. 3 67	3	none	ext. 165 flex. 30°	normal flex. and ext.
7	right knee	10. 4 67	3	none	flexed t 45°	normal flex. and ext.
8	right knee	19 6. 67	1 bed ridden for 3 months	none	ext. 165° flex. 30°	normal flex. and ext.
9	right and left knee	19 6. 67	right: continuous bleeding	none	flexed at 20°	normal ext., flex. 60°
			left: 2	none	ext. 165° flex. 70°	normal ext., flex. 40°
10	right knee	26. 6. 67	2	none	flexed at 90°	normal ext., flex. 50°
11	right knee	28. 6. 67	2	none	normal ext., flex. 60°	normal flex. and ext.
12	tibio-tarsal joint	13. 3. 68	continuous bleeding	none	strict joint	normal joint
13	left knee	28. 10. 68	1 bed ridden for 4 months	none	ext. 160° flex. 95	normal ext., flex. 95
14	left knee	11 11 68	continuous bleeding	none	ext. 150° flex. 90°	normal ext., flex. 90°

flex. = flexion, ext. = extension.
postoperative period less than 1 year

even though restricted in length, allowed easy exploration of the cavity under the quadriceps femoris and of the small cavities under the fat pad and on the sides of the patellar ligament. The technique practiced for removal of the deep synovial membrane layer was that used in normal synovectomy

Surgical haemostasis was impossible because of the strong adherence of the deep layer to the fibrous layer of the synovial membrane. This particular situation caused diffuse haemorrhages which flooded the operating surface, which were very difficult to control in the first two cases (table I) In the other cases, the use of antifibrinolytic compounds (Frey parotid inhibitor) on the bleeding surface gave rapid and complete control of the situation.

All patients received Ampicillin (34 mg/kg/day) from the time of operation until the 10–15th postoperative day (until the 40th postoperative day in the first and second case)

Gross Pathological Characteristics of Operated Articulations

For simplicity even though this may appear arbitrary we have divided the strata (from the interior proceeding toward the exterior) of the deep surface of the articular cavity into superficial, intermediate or basal and fibrous.

In the 16 operated articulations we have grouped the gross appearance of the superficial layer in 3 fundamental types 1 hypertrophic-angiomatous, 2. hypertrophic-pigmentous, 3. hypertrophic-fibrous. It is possible that the histological study of the excised tissue will modify this classification.

In articulations of the hypertrophic-angiomatous type, the superficial layer was covered by varicose vein formations of diverse appearance. These were globular nodular serpentine or resembled leaf-like formations. Their size ranged from millet grain to grape. The walls of these formations were thin and delicate. Merely touching them with gauze caused laceration and the appearance of fluid resembling blackberry jam in colour and consistency These formations adhered to layer of fragile, loose connective tissue of grayish-yellow colour which was easily removed by retention curettes. In some parts of the articulation (posterior surface of the quadriceps femoris) the connective tissue was thicker more consistent and strongly adherent to the underlying fibrous stratum.

Articulations of the hypertrophic-pigmentous type were characterized by the involution of the plexus-like angiomatous formations described in the preceding type. These formations were smaller did not contain blood and were of colour which ranged from brick red to tobacco brown and in some cases also sepia. Their shape was variable, ranging from spherical to ovoid, and in some cases they were thready and even assumed the appearance of thin beard or marine algae. The stratum to which these structures adhered was of an ivory-yellowish colour It was more consistent than the basal layer of type 1. Few villi were noted. Adherence of the basal or intermediate layer to the deep fibrous stratum was more tenacious than in the preceding group.

In articulations of the hypertrophic-fibrous type, the fibrous and angiomatous formations which formed the superficial stratum in the 2. and 1 types, were no longer evident. Instead, consistent yellowish islands on the intermediate or basal layer predominated. Scattered points of brown pigment residue were observed. The basal layer showed strong structural affinity to the underlying fibrous coat and was united wit it in several areas. Synovial membrane removal was more difficult than in the preceding types.

The 3 types of synovial membrane alteration probably correspond to progressive stages in the evolution of haemophilic arthropathy For each we noted corresponding changes in the bone of the joint and the articular cartilage.

Table III. Haemorrhages in other sites (articular and other not including site of operation)

Case	Previous to synovectomy
1	Continuous in left elbow left tibio-tarsal joint, left shoulder painful, absorbed in 15 days in muscles of the calf bilat. Numerous and frequent cutaneous ecchymoses.
2	Continuous at the left elbow voluminous, fever painful, absorbed in 8–10 days. 15 serious epistaxes.
3	5 t the right knee, painful, fever cast applied, ankylosis of the articulation. Frequent and numerous cutaneous ecchymoses.
4	Continuous at both elbows, voluminous, painful, absorbed incompletely 1 hematuria.
5	Continuous in left knee, at both tibio-tarsal joints and elbows very frequent in the muscles, with immobility and pain. 7 epistaxes 2 hematurias.
6	Continuous at the left knee, both elbows, both ankles 1 at the right hip, absorbed in 10–15 days, at the coxa in 25 days.
7	2 serious episodes of hematuria. Numerous and frequent cutaneous ecchymoses.
8	3 at the right quadriceps, voluminous, painful, absorbed in 15 days.
9	Continuous in ankles and elbows, violent, pain, incomplete absorption.
10	1 gingival caused by trauma. Frequent cutaneous ecchymoses.
11	Frequent cutaneous ecchymoses.
1	Continuous t left tibio-tarsal joint; 1 t right elbow voluminous and painful. 3 epistaxes.
13	Continuous t right tibio-tarsal joint 4 at both elbows.
14	*Continuous at left tibio-tarsal joint and at left elbow*

Postoperative period less than 1 year

In articulations with angiomatous hypertrophy of the synovial membrane (type 1) the cartilage was thin, fragile and lacked the mother-of-pearl lucidity characteristic of healthy cartilage. However it could be considered essentially normal. The epiphyses appeared less consistent than *the epiphyses of normal children at comparable age but could not be* considered abnormal.

In the articulations with pigmentous hypertrophy of the synovial membrane (type 2) we noted large islands with irregular borders of cartilagenous destruction. These were localized on the posterior surface of the rotula and on the femoral condyles. A few were found on the tibial plateau. A large area of compressed, greenish-yellow spongy bone replaced the cartilage.

The periosteum was lacking on the epiphyses where the cartilage ended or was reduced to a thin non-vascular layer This condition extended to the bone shaft.

occurred in 1 year Fresh whole blood or fresh plasma employed in 1 year

After synovectomy	Fresh whole blood (F bl.) or plasma (F pl.) previous to synovectomy		after
5 t the right tibio-tarsal joint and at the left	F bl.	2,150 ml	none
elbow modest, painless, spontaneous	F pl.	1,800 ml	none
absorption in 3 days			
2 t the left elbow modest, painless,	F bl.	900 ml	none
absorbed in 3–4 days			
none	F bl.	1,250 ml	none
	F pl.	1,550 ml	none
none	F bl.	2,500 ml	none
	F pl.	4,000 ml	none
1 at left tibio-tarsal joint, 4 at both elbows,	F bl.	3,000 ml	none
modest, painless			
2 at the right deltoid muscle, modest,	F bl.	1,800 ml	none
caused by trauma, bsorbed in 7 days	F pl.	1,500 ml	none
none	F bl.	750 ml	none
	F pl.	750 ml	none
none	F bl.	900 ml	none
	F pl.	750 ml	none
none	F bl.	4,500 ml	none
	F pl.	3,200 ml	none
none	F bl.	1,200 ml	none
none	none		none
none	F bl.	350 ml	none
	F pl.	700 ml	none
none	F bl.	1,500 ml	none
	F pl.	700 ml	none
none	F pl.	2,800 ml	none

In articulations with fibrous hypertrophy of the synovial membrane (type 3) the cartilage under stress had disappeared. The underlying yellowish bone appeared more compact and smoother than in the preceding type due to compression of the bony trabeculae.

Therapeutic Scheme of Haemostasis

I the 2 earliest synovectomies (table I cases 1 and 2) haemostasis was obtained by the administration of fresh frozen plasma, continued until the 35th postoperative day (18–20 ml/kg/day in 2 daily transfusions) Coagulation time and prothrombin consumption test (table IV) remained normal throughout the 35 day period however blood collected from the synovectomized articulation at different postoperative intervals did not coagulate and at most formed loose and fragile clots.

Table IV Whole blood clotting time (CT) and prothrombin consumption test (PC) during postoperative period

Case	Before operation		1 day (operation)		3. day		6. day		10. day		15. day		20. day		30. day		45. day	
	CT min	PC sec	CT min	PC sec	CT min	PC sec	CT min	PC sec	CT min	PC sec	CT min	PC sec	CT min	PC sec	CT min	PC sec	CT min	PC sec
1	> 60	8	7	> 60	8	> 60	7	> 60	7	> 60	7	> 60	6	> 60	10	> 60	> 60	8
2	> 60	4	8	> 60	7	> 60	9	> 60	7	> 60	7	> 60	8	> 60	9	> 60	> 60	4
3	35	6	9	> 60	10	> 60	10	> 60	10	> 60	9	> 60	11	> 60	35	6		
4	20	8	9	> 60	10	> 60	9	> 60	8	> 60	9	> 60	11	> 60	20	8		
4	20	8	7	> 60	10	> 60	8	> 60	8	> 60	20	8						
5	> 60	5	10	> 60	11	> 60	9	> 60	> 60	5								
6	> 60	6	9	> 60	33	8	> 60	6										
7	> 60	6	8	> 60	8	> 60	13	> 60	> 60	6								
8	12	28	6	> 60	6	> 60	8	> 60	10	> 60	12	28						
9	> 60	5	8	> 60	8	> 60	8	> 60	11	> 60	12	> 60	7	> 60	8	> 60	> 60	5
10	> 60	7	6	> 60	5	> 60	6	> 60	6	> 60	5	> 60	6	> 60	9	> 60	> 60	7
11	30	10	8	> 60	9	> 60	8	> 60	8	> 60	10	> 60	30	10				
12	> 60	7	7	> 60	9	> 60	> 60	7										
13	> 60	8	6	> 60	11	> 60	31	65	> 60	8								
14	> 60	5	6	> 60	7	> 60	> 60	13	> 60	5								

Only from the 23–28th day after surgery did blood from the articulation coagulate. At that time (32 35th day), we obtained large, formed or moulded clots through openings in the surgical wound.

In the remaining 14 synovectomies the haemostatic scheme was very different

1 Parotid inhibitor (Trasylol®) was introduced into the articulation during the final stages of the operation. The dose varied from 100,000 to 500,000 UIC according to the operation surface. Parotid inhibitor was again introduced into the joint, usually in doses of 25,000 UIC when articular pain was noted in the postoperative period. Pain was always accompanied by elevated local temperature, redness and edema of the wound and skin surrounding the joint.

2. Epsilon-amino-caproic acid (EACA) intravenously (in solution of 250 ml of 5% glucose) and *per os*. The standard dose was 0.3 g/kg/day but was increased to 0.9–1.0 g/kg/day in some cases. The daily dose of EACA varied according to the objective and subjective symptoms observed in the operated joint. EACA administration began one day before surgery and was continued for 3–4 weeks.

3. Fresh frozen plasma was usually employed in doses of 8–10 ml/kg/day The duration of administration did not exceed the 10th postoperative day Plasma administration always began one day before surgery

This haemostatic programme was most efficient, strongly contrasting to the results in the 2 earliest cases, in which only fresh frozen plasma and fresh whole blood were employed. This scheme allowed mobilization of the articulation from the 12–15th postoperative day No toxic side effects resulted from the use of EACA even though it was sometimes employed in massive doses.

Comments on Haemostatic Therapy

It has been known for a long time that the haemophilic patient could successfully undergo abdominal surgery (as we have also seen in other cases) with the protection of fresh plasma, fresh frozen plasma or plasmatic fractions. However operations involving musculoskeletal organs were often followed by a long and complicated post-operative period because of frequent and severe haemorrhages at the operation site [3 12, 13] This difference has been confirmed by the clinical signs observed in the postoperative period of our first 2 cases (table I) In these 2 patients the elevated intraarticular pressure due to continuous haemorrhage caused necrosis of the marginal skin of the surgical wound. To reduce this pressure, arthocentesis was attempted several times. This was inefficient and dangerous, because we always obtained blood under strong pressure. In this situation, which at times was dramatic, we were forced to apply a knee cast in extension. Later this cast was opened on the superior surface of the knee joint and finally reduced to a water leader type support which gave satisfactory results.

Since in our first 2 cases the administration of plasma did not prevent intraarticular haemorrhage, we were convinced that further

synovectomies would not be possible, unless the problem of haemostasis could be solved

The fact that articular bleeding continued for a long period and with violent fits, even though clotting time and prothrombin consumption test remained normal, led us to postulate that the protracted intra-articular haemorrhage could be due to particular tissue conditions, which could produce unfavourable effects on local haemostasis in subjects with an already compromised haemostatic balance.

The above hypothesis was based on the clinical findings in our first 2 cases and on the studies of ASTRUP *et al.* [14, 15] dealing with the thromboplastic and fibrinolytic activity of the human synovial membrane and fibrous capsule and the fibrinolytic activity of connective tissue in various stages of aseptic repair

These authors obtained the following results 1 absence in the synovial membrane and in the fibrous articular capsule of tissue thromboplastic activity and presence of fibrinolytic activity 2. development of elevated fibrinolytic activity in granulation tissue in fibrous evolution (peak from second to fourth week).

The clinical phenomena which we observed in our first 2 cases would appear to agree with the above conclusions. In both patients we noted, during the course of protracted haemorrhage, two violent intra-articular bleeding episodes, the first of short duration in the early days following the operation, the second, more serious, began toward the 8 or 9th day and lasted until the fourth postoperative week, coinciding exactly with the granulation stage.

Our hypothesis seems confirmed by the excellent haemostatic results obtained in the remaining cases (all severe haemophiliacs) which were treated by intense systemic and local antifibrinolytic therapy in addition to the traditional plasma therapy Thus therapeutic combination greatly reduced the total amount of plasma required The daily dose of antifibrinolytic compound varied according to the subjective and objective signs at the articulation site, namely fever intense pain, edema and redness of the surgical wound and of the skin surrounding the articulation. These symptoms regressed immediately when the dose of EACA administered i v or *per os* was increased (from 0.3–0 4–0.5–0 6 etc. g/kg/day) or when the antifibrinolytic compound was directly introduced into the articular cavity (parotid inhibitor 25 000 UIC)

Using the above haemostatic scheme we have had no haemorrhagic complications and the postoperative period, especially in the more recent cases, has been comparable to the postoperative period after knee synovectomy of patients with normal haemostasis.

The effect of antifibrinolytic compounds on postoperative haemostasis in the synovectomized joint of haemophiliac, was particularly evident in case 6, patient with haemophilia A. In this patient the whole blood clotting time lengthened (30 h after surgery the whole blood clotting time was no longer measurable) even though he received continuous massive doses of fresh frozen plasma. This severe condition was probably due to sensitization toward factor VIII. Therefore plasma administration was stopped, treatment was continued only with antifibrinolytic compounds. The fact that no bleeding occurred must be attributed to the use of the latter

Conclusions

We believe that surgical treatment of haemophilic arthropathy may be a new and valuable therapeutic approach. From our preliminary experiences we may draw the following conclusions

1 To date there has been no recurrence of haemarthrosis in the 16 synovectomized articulations. Marked functional improvement in the joint is noted and in some cases this is confirmed by the radiological appearances.

2 All synovectomized patients show a marked decrease in the number and gravity of haemorrhagic episodes both in other articulations and in extraarticular sites. Rare haemorrhagic incidents produced little pain. Furthermore it was not necessary to give fresh whole blood or whole plasma or plasma fractions, in contrast to the pre operative requirements. The improvement of our haemophilic children following synovectomy is further corroborated by a marked decrease in school absence and the fact that the children are able to engage in games from which they had previously been excluded.

3 A haemostatic procedure enables us to treat haemophilic articulations surgically without haemorrhagic complications. It consists of the simple association of intense antifibrinolytic therapy (system and *in loco*) and a relatively small amount of fresh frozen plasma.

4 The satisfactory results obtained lead us to believe that at present synovectomy may be the best treatment for haemophilic arthropathy We feel it should be tried also on the elbow and tibiotarsal articulation (for this last articulation we have had only one case) We also suggest that synovectomy may be performed as early as possible, preferably immediately after the recurrence of haemarthrosis in the same joint and before serious bone and cartilage alterations occur

Our observations support the hypothesis that florid haemophilic arthropathy exerts an unfavourable influence on the number and

severity of haemorrhagic incidents. Considering the efficiency of antifibrinolytic therapy on haemostasis during knee synovectomy in serious haemophiliacs and the favourable results obtained in the articulation and the decrease of haemorrhagic episodes in other sites, it seems reasonable to assume that excision of hypertrophic synovial tissue removes from the haemophiliac a rich source of tissue activators of plasminogen. The presence of this hypertrophic synovial membrane could have unfavourable effects on the seriously compromised haemostatic balance of the haemophiliac. In haemophiliacs undergoing synovectomy in the near future, the coagulation and fibrinolytic pictures (including those at the level of the operated articulation) will be studied more completely as a control of the above hypothesis.

Summary

The authors report 16 synovectomies (15 of the knee and one tibio-tarsal) performed on patients with severe haemophilia A or B. The macroscopic anatomo-pathological picture of the synovectomized articulations and the haemostatic therapy during the postoperative period are described. The excellent results, local and general, indicate synovectomy as a new and precious therapy of haemophilic arthropathy

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Addendum

At the present time (20 6. 1969) the synovectomies performed are 19 on 16 patients.

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Prophylaxis of Joint Hemorrhages in Hemophilia

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It is a well-known fact that many hemophiliacs A and B suffer from more or less severe arthropathia haemophilia, as a result of recurrent joint hemorrhages. The question whether this arthropathia might have been prevented does not apply to patients aged between 20 and 30 years or older, as the better insight into the treatment of joint hemorrhages in hemophilia only dates from the last 10 to 15 years, and joint hemorrhages, especially in large joints, usually only start to appear when the child begins to walk. Kerr [1] has ascertained that 75% of all children with severe hemophilia had at the age of 4 years already suffered at least once from a hemarthros. At the age of 10 years 80% already showed clinical signs of pathologic changes in the knees and at the age of 16 years 75% was crippled and showed hemorrhages in other joints. These figures conform to those of others and to our own.

As far as the older patients are concerned we may assume that at the time they had their first joint hemorrhages no such therapy existed which might have prevented severe malformations. This in spite of the fact that more than 30 years ago it had already been established in the Netherlands [2] that in classic hemophilia there is a deficiency of one of the clotting factors in the plasma. However, at present the situation is different. It is now more or less generally accepted that every acute joint hemorrhage in hemophilia should be treated promptly, meaning preferably as soon as the patient himself feels the hemorrhage coming on [3]. This treatment includes rest, elevation of the stricken extremity

[1] With the technical assistance of Mrs. C. J. M. McEwan-van der Horst and Mrs. D. L. Frere Voortelman.

immobilisation of the joint, icepacks, transfusion and perhaps puncture of the joint.

It is generally accepted that treatment of an acute joint bleeding with transfusions of fresh plasma, or fresh frozen plasma, or of a concentrated plasma product is indicated. The same applies to the immobilisation of the joint. But there is no agreement regarding joint puncture in acute hemorrhages. Some physicians always prefer to puncture after a preceding plasma transfusion and possible subsequent ones, others never or hardly ever perform a puncture. But even experienced doctors such as KERR who has performed punctures in hundreds of knee-joints in acute hemorrhages, stress the dangers of such punctures in hemophilia, of which we here mention only infection of the joint, and provocation of a new bleeding. In addition the enthousiastic supporters of puncture treatment in bleedings into the knee-joint have to admit that punctures in other joints may give rise to serious damage. Some questions now arise:

1. Can the joint with an acute hemorrhage be emptied of blood by means of a puncture? Often this is not the case, even when the joint is easily accessible. In a large number of punctures KERR was able to remove 0–170 ml (average 25 ml). Other investigators also give greatly varying quantities of aspirated blood, especially when puncturing repeated joint bleeds. But the remaining blood will undoubtedly produce changes in synovia, joint capsula and in the long run also in the joint cartilage, in the same way as when no puncture is performed.

2. Can systematic puncture and transfusion prevent the danger of arthropathia haemophilica? Neither by the therapy of prompt transfusion with plasma or AHF-concentrate, which we prefer, nor by puncture(s) combined with transfusion(s) can the danger of severe or less severe arthropathia be entirely eliminated. The risk can, however, be greatly diminished by such treatment. Two of our patients who had been under our care for a long period, and who every time were promptly treated for each joint hemorrhage by transfusions died after a car accident. The pathologist was able to study one or more joints, and in both cases important changes were found in the cartilage and in the underlying bone.

3. Does the systematic treatment with transfusions of plasma or AHF-concentrate (with or without puncture of the joint) not involve the risk of furthering the development of a circulating anticoagulant? And can this risk be reduced by regular puncturing of a bleed, and

then giving the smallest number of transfusions? The inhibitor is usually acquired before the age of 10 and the percentage of severe hemophiliacs A in whom circulating anticoagulants were found varies greatly [4]

A second risk of the application of transfusions of plasma and plasma products is the appearance of serum hepatitis, a danger which some investigators consider to be very small, while others mention a high frequency From our experience we consider the danger of hepatitis to be very small, but the plasma which we use is each time obtained from only one donor and the cryoprecipitate from 4 donors.

We have applied punctures in acute joint bleedings only a few times and at these occasions we were not all convinced that the joint had been completely emptied of blood The risk of a hemorrhage in joint puncture is also not unimaginary if we bear in mind the blood vessels in the synovia which can be damaged in this way and the hyperaemia of the synovia in such joint bleeds.

When a patient suffers from very frequent joint bleeds and if, in spite of prompt transfusion therapy of each hemorrhage, the first signs of arthropathia haemophilica have already appeared another question arises whether in those cases prophylactic treatment is still possible and whether the risk of severe arthropathia haemophilica with all its medical and social consequences (a.o educational) should prevail over the danger of the appearance of a circulating anticoagulant. In 2 such patients we have had to answer this question in the affirmative.

Already many years ago it was recommended in cases of repeated hemorrhages in hemophilia A to try and prevent these bleeds by regular plasma transfusions, amongst others by JOHNSON [5] and by ALEXANDER and LANDWEHR [6] in hemophilia A, and recently by KING [7] in hemophilia B. Of late in hemophilia A concentrated plasma products have been used for this purpose [8, 9 10]

Among the large number of young hemophiliacs A whom we have treated from the moment they suffered their first joint bleeds there are several who so frequently got joint hemorrhages that they needed almost constant therapy in order to prevent further crippling Two boys already showed the beginning of arthropathia haemophilica (the boy K.T in particular) and in the long run a marked damage of the joint and a chronic crippling joint disease accompanied by all its medical and social consequences (a.o. educational) was to be feared. We decided to try to prevent joint bleeds completely in these 2 children.

Through the particular co-operation of Dr H. W Krijnen the Central Laboratory of the Netherlands Red Cross allowed us sufficient freeze-dried cryoprecipitate Both these children lived in the vicinity of the Hemophilia Clinic. They could therefore return regularly for their transfusions while continuing to attend the schools. We deliberately gave these children these transfusions, as we considered for them the danger of becoming crippled to be much greater than that of developing a circulating anticoagulant. We started from an amount of cryoprecipitate which when used in previous acute hemarthroses had repeatedly resulted fairly quickly in a decrease of the joint bleed. Initially we gave 3 transfusions a week, because already after 48 h the AHF-level in the plasma had returned to the original level (less than 1%) This was occasionally checked by means of a so-called consumption curve. After some weeks the amount of cryo was gradually decreased in order to be able to determine which was the smallest amount of cryo required to prevent the joint bleeds. At present (February 1st, 1969) the first patient (H.) has been receiving this prophylactic treatment since November 1st, 1967 the second one (K.T) since February 1st, 1968.

Each cryo is prepared from 1 000 ml plasma and before transfusion dissolved in 50 ml water

Case Reports

Case 1 Patient H. was born on 24.11 1957 He had severe hemophilia A, bloodgroup O Rh+ Between 20.12.1964 and 15.11 1967 he was admitted to the Hemophilia Clinic many times on account of acute joint bleedings and also treated frequently for hemorrhages into muscles. As result of the recurrent knee hemorrhages the right leg had become 1 cm shorter than the left, the left knee was swollen and on both sides there was valgus position of the leg Special orthopedic shoes and surgical iron were prescribed for him. Since the beginning of November 1967 the boy has been treated prophylactically as described in figure 1.

At first he was given transfusion of 2 freeze-dried cryo 3 times week. On 25.12.1967 he had joint complaint for the first time since the prophylactic treatment was started; the left knee was painful, slightly swollen and warm. No special measures were taken except ordering him to rest for day and on 27 12.1967 the complaints had disappeared. From 24.2.1968 he was given only one cryo thrice weekly On 27.2.1968 the left ankle was painful and slightly swollen, but the following day there were no more complaints, neither subjectiv nor objective. On 4.3.1968 there were again shortlasting complaints of the right ankle without swelling. With the idea in mind that these complaints were due to slight hemorrhages we gave 1 ½ cryo thrice week. When on 1.4.1968 the condition had remained

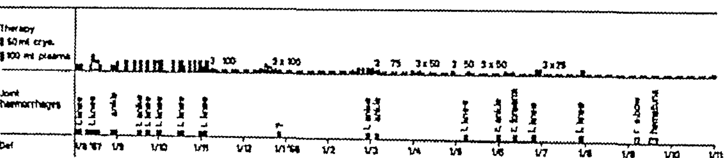


Fig 1 Case 1 Joint hemorrhages, indicated by black dots, occurring before the prophylactic transfusions were started (1 8. 1 11 1967). From 1 11 1967 until now prophylactic therapy with cryoprecipitate has been applied. Only the transfusions given from 1 11 1967 1 10 1968 are indicated. No joint hemorrhages were observed during this period, only occasionally slight complaints of joints, indicated by hatched blocks. 3 (2) × 100 (75, 50, 25) = 3 (2) × per week 100 (75, 50, 25) ml cryoprecipitate.

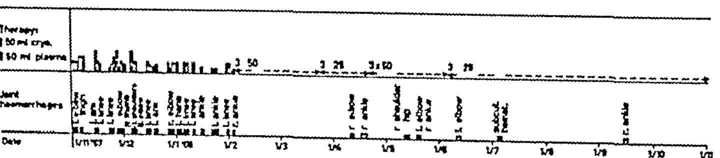


Fig 2 Case 2 Joint hemorrhages before and after prophylactic transfusions. Legends as in figure 1

quite satisfactory w again gave one cryo thrice weekly On April 27 1968 we started with one cry twice week. However when on May 6th slight complaints of the left knee occurred, from May 8th w again gave one cryo thrice weekly From time to time these slight joint complaints re-appeared. Nevertheless, from June 8th, 1968 we gave ½ cryo thrice week. This was continued until November 1st, 1968. Occasionally there were slight complaints of joint lasting one day During the entire period from November 1st, 1967 with the exception of few days, the boy went to school regularly whereas formerly he had to be absent from school for many weeks each year.

The experience between November 1th, 1968 and February 1st, 1969 is with thrice weekly one freeze-dried cryo there are no complaints, with thrice week ½ cryo occasionally very slight joint complaints.

By giving him thrice weekly one freeze-dried cryo the boy would receive in one year 52 × 3 cryo's. Assuming that one cryo contains 40% of the AHF present in 1,000 ml plasma, this would mean 52 × 3 × 400 = 62,400 units AHF And when given 3 times week half cryo, this would mean 31,200 units AHF per year During the year preceding the beginning of the prophylactic treatment pat. H. received 32,847 units AHF However during this preceding year the boy was absent from school for several weeks and there was no complete prevention of damage to the joints.

Case 2. Patient K.T was born on 6.6.1958. H had severe hemophilia A, bloodgroup B Rh Between 3.8.1964 and 10.12.1968 he was admitted to the Hemophilia Clinic 17 times for joint bleeds and in addition was treated frequently for hemorrhages in muscles. Between 1.2.1967 and 1.2.1968 he was given many transfusions of plasma and freeze-dried cryoprecipitate on account of these hemorrhages. Also for this boy special orthopedic measures were prescribed.

From 10.2.1968 this boy was treated prophylactically at first for several weeks clinically later on polyclinically only while attending school regularly (fig 2) On February 10th, 12th, 13th and 14th he was given one cryo every day from February 16th 3 times week one cryo, and from March 18th thrice week ½ cryo. Since then he occasionally had shortlasting complaints about some joints without swelling and change of temperature. At first we used to increase temporarily the quantity of cryo after such complaint, but from June 6th until September 15th the dose of half cryo thrice weekly was maintained.

A so-called consumption curve was made from time to time in both patients. From these curves it appeared that with one cryo as well as with half cryo the AHF content of the plasma had decreased to low value or to less than 1% 24 h after transfusion. Based on transfusion frequency of ½ cryoprecipitate thrice weekly until yet, this would mean that he was receiving 1½ l of plasma weekly or 52 × 1½ = 78 l yearly Assuming that the AHF alue of the cryo is 40% (it varies between 35–50%) this would mean total of approximately 31,200 units AHF per year In the preceding year he received 20,088 units AHF So this patient got much more AHF prophylactic treatment than in the preceding period for therapeutic purposes however this boy too could now go regularly to school.

From October 11th, 1968 until December 12th, 1968 this patient was given only twice week *transfusion of ½ free-dried cryoprecipitate and he remained free from joint* symptoms. Between December 12th, 1968 and January 6th, 1969 he had to be hospitalized on account of serious infection after trauma. Since then the prophylactic treatment was resumed and the recent experience till February 1st, 1969 is that with thrice week ½ cryo the boy is without complaints, while with twice weekly ½ cryo he occasionally has slight complaints. Three times weekly ½ cryo would mean yearly 3 × 200 × 52 = 31,200 units AHF and twice weekly ½ cryo 2 × 200 × 52 = 20,800 units AHF In the year before the prophylactic treatment started this boy had got in total (plasma and cryoprecipitates) 25,465 units AHF and he was unable to go to school for many weeks.

Discussion

How long should this prophylactic treatment be continued? In cases of severe hemophilia A and B aged younger than 5 years chronic abnormalities of the joints usually still are absent. But, if these children are not treated after that age the deformities will become more frequent and more severe. Often around the age of 15–20 years there is a tendency to a decrease in frequency of joint hemorrhages. We should like therefore at least until that age to treat each of these children prophylactically. However it hardly needs explaining that at present this prophylactic treatment is impossible on a larger scale on account of the very large quantities of fresh plasma or concentrate required.

The regular intravenous injections in both our patients never gave rise to difficulties and in this respect concentrated AHF products have also an advantage compared to the much larger quantities of fluid required in giving plasma transfusions. However the quantity of plasma needed for efficient prophylaxis greatly varies from patient to patient. The fact that an intravenous injection had to be given 2–3 times a week is of course a drawback, although as said before, in our 2 patients it gave no difficulties. With more concentrated factor VIII products, requiring for their preparation much larger volumes of plasma, it might be possible to reduce the number of transfusions to one per week [9, 11, 12]. But with such more concentrated products the risk of serum hepatitis is probably also greater.

It was not always possible to use freeze-dried cryoprecipitates on a type-specific basis. There is, however, evidence that no concentration of iso-antibodies occurs during cryoprecipitation [13]. In patient H. with bloodgroup O Rh+ the titres of anti A and anti B agglutinines in his serum were determined after he had been getting prophylactic transfusions for one year. The Central Laboratory of the Dutch Red Cross found for the anti-A and anti B agglutinines a titre of $^1/_1$ and $^1/_4$ respectively. In patient K.T. with bloodgroup B Rh– the titre of iso-agglutinines anti A in the serum, 9 months after the beginning of the prophylactic treatment, was $^1/_8$.

On January 15th, 1969 the fibrinogen content of the plasma was determined, which determination was of importance after so many transfusions of a fibrinogen-rich product had been given. In patient H. we found a value of 300 mg% and in patient K.T. of 240 mg%. On that same date various liver function tests gave a normal result.

Five, 8 and 11 months after beginning the prophylactic therapy in the first period we made control X rays of both boys. We were aware of the fact that particularly in the second boy the duration of the prophylactic treatment was still short, but in both patients the arthropathia showed no deterioration of importance.

Conclusions

We definitely feel that by giving the 2 patients with hemophilia A the described prophylactic treatment we have succeeded in preventing the damage to the joints from progressing further and further to the stage where crippling would develop. Before this prophylactic treatment was started we had given early replacement therapy i.e. each time when a joint hemorrhage occurred. However we could not prevent damage to the joints in this way and moreover the children had to be hospitalized fairly frequently

With others we agree that such prophylactic treatment is not possible on a somewhat larger scale, amongst other reasons because we do not have sufficient material, and also on account of the danger of developing circulating anticoagulants. Prophylactic treatment as applied in our 2 cases will also not always be necessary However notwithstanding very early treatment of acute hemarthroses we do not know in which cases the joint bleedings are the most damaging Individual judgment of the cases will therefore always be necessary The now existing possibility of completely preventing crippling in cases where, in spite of very early treatment, progressive damage of the joints occurs, means great progress in every respect.

Summary

A long-term prophylactic study was made in 2 boys, aged 11 and 10 years, suffering from severe hemophilia A, who both had already had many joint hemorrhages. Starting with transfusion of 3 'freeze-dried' cryoprecipitates 3 times week we gradually decreased to 3 times week half of freeze-dried cryo. With this prophylactic treatment one patient had been without distinct joint hemorrhages for 14 months and the other one for 12 months. Both could lead normal life and they regularly went to school during all this time.

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Different Blast Kinetics in Acute Myeloblastic and Lymphoblastic Leukaemia

A Hypothesis of Different Stem Cell Origin

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Increasing data on cell proliferation kinetics in acute leukaemia has led to emphasize a different blast behaviour in acute myeloblastic leukaemia (AML) and acute lymphoblastic leukaemia (ALL) In previous *in vitro* studies we observed a certain number of ALL cases in which the peripheral blood proliferative activity was significantly higher than that of bone marrow cells and was accompanied by a corresponding difference in proliferating compartment size [1] Following a single administration of thymidine in a case of ALL, we were able to evaluate the size of the proliferating and non-proliferating compartments and to show a difference of the growth fractions of the marrow and of the peripheral blood [2] this result contrasting to the usual picture in AML [3–11] Consequently a prevalent extramedullary origin of lymphoblastic leukaemia cells was suggested.

The present research has been carried out to throw further light on kinetic differences between ALL and AML populations and to see whether the differences in blast kinetics indicated a real dissimilarity in the stem origin of the 2 forms of acute leukaemia. The recognition of the precursor cells in acute leukaemia is of crucial importance with respect to the histogenesis of leukaemia in man [12–14] They are probably the site of the first leukaemic transformation and are, as feeder cells, responsible for the development of the blood picture associated with the disease. Direct identification of leukaemic stem cells in morphological terms is as yet out of question. Their behaviour

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can however be mapped out via detailed evaluation of proliferating compartment blast kinetics. Theoretically one can assume that stem cell kinetics will lead to kinetics in the next following compartment, i e, that of the proliferating blasts [15–17]

In the present studies an analysis of the kinetic behaviour of the cell population in function of blast diameter has been made in cases of ALL and AML after both single and repeated *in vivo* pulse labellings with ^{3}H thymidine The latter procedure secured the labelling of a larger proportion of the blast population whose kinetics and eventual fate could be studied for a sufficiently long period after labelling

Materials and Methods

Three cases of ALL (No. 1 2 and 3) and 2 cases of AML (No. 4 and 5) all untreated and with stationary clinical and haematological pictures, were studied.

In case 1 a single dose of 0.64 $\mu c/g$ ^{3}H-thymidine (specific activity: 9 c/mM) was injected i.v. Bone marrow samples were taken 1 14 24 72 h and 5 days after the thymidine injection.

Case 2 received single dose of 0.25 $\mu c/g$ ^{3}H-thymidine (specific activity 2 c/mM). Marrow blood samples were taken 1 18, 66 h and 7 days and peripheral blood samples up to 7 days after the thymidine injection.

Case 3 received 4 × 8-hourly ^{3}H-thymidine (specific activity: 2.2 c/mM) injections (to total amount of 0.73 $\mu c/g$). Marrow blood samples were taken 9 25, 49 73 h and 5 days and peripheral blood samples until the 7th day after the first injection.

In case 4 a single dose of 0.26 $\mu c/g$ ^{3}H-thymidine (specific activity: 24.8 c/mM) was injected i.v. Bone marrow samples were taken 1 10, 26, 72 h, 5 and 8 days and peripheral blood samples up to 8 days after the thymidine injection.

Case 5 received 4 × 10-hourly ^{3}H-thymidine (specific activity 2 c/mM) injections (to total amount of 0.42 $\mu c/g$) Marrow blood samples were taken 2, 30, 54 78 h, 5 and 7 days and peripheral blood samples up to 7 days after the first injection.

Standard techniques were used for the preparation of autoradiographic marrow and peripheral blood smears [18] These were exposed at 4 °C for 120–150 days.

Labelling index (LI) values were determined on at least 1,000 cells and mean grain counts (MGC) on at least 200 labelled cells. Labelled and non-labelled mitosis counts were made on at least 50 figures at each time interval. Marrow and peripheral blood blast populations were subdivided into 3 equal classes according to diameter

Results

Single injection of ^{3}H-thymidine In case 1 the marrow blast population was composed of 13 % large 47 medium and 40% small cells (assessed on the basis of 3 equidistant diameter steps) These percent values remained constant throughout the experiment.

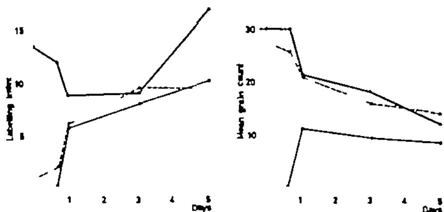


Fig. 1 LI and MGC of the large (——), medium (-----) and small (————) blasts in bone marrow in case of ALL (case 1)

Bone marrow

Peripheral blood

Fig. 2 LI and MGC of the large (——) medium (——) and small (————) blasts in bone marrow and peripheral blood in case of ALL (case 2)

One hour after thymidine, LI values were 13 0.9 and 0% for large medium and small cells respectively (fig 1) At the 24th h, large cells had decreased, medium cells had increased and labelled small cells were observed for the first time. By the 5th day large cells had increased sharply to higher than start levels and there were further increases in medium and small cell values.

Large cell MGC values fell in the first 24 h and continued to decrease though more slowly up to the 5th day Medium cells showed a similar trend whereas small blast MGC's remained virtually unchanged.

Population percents were 15 % large, 40% medium and 45% small in case 2 LI values of 20.7 (large blasts) 2.2 (medium blasts) and 0 (small blasts) were observed at the end of 1 h (fig 2) Large cell LI increased to 33.3 by the end of the 18th h followed by a fall to 14 4 by the 66th h the medium cell LI value also increased, whereas the first labelled small cells were not observed till the 18th–66th h. A sharp fall in large and medium cell MGC values was observed between the 1st and the 66th h.

A considerable number of labelled blasts were counted in the peripheral blood after 1 h, without distinction of cell classes the whole population LI was higher than in the marrow (20.1 versus 3 4) Circulating blast LI values are shown in figure 3 The same diameter classes were used as in marrow cells and the percent distribution was about the same. However the LI behaviour was different large cells showed a decrease between the 1st and 18th h (as opposed to the

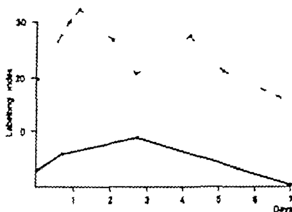


Fig 3. LI of the whole blast population in bone marrow (——) and peripheral blood (------) in case of ALL (case 2)

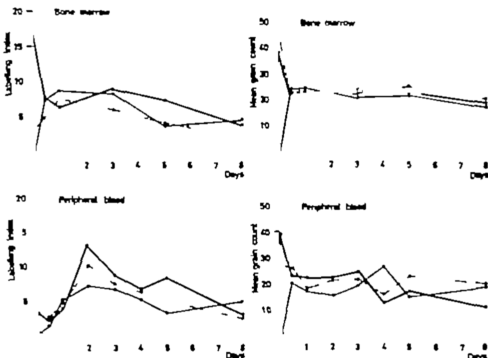


Fig 4 LI and MGC of the large (——) medium (-----) and small (————) blasts in bone marrow and peripheral blood in case of AML (case 4)

increase of the large marrow blasts) followed by variable but continuously high levels (30–45) falling only after the fifth day (fig 2) Medium blasts displayed a sharp rise in the first 24 h and were accompanied by labelled small cells (absent in the first hour) Within the first 48 h, large and medium MGC values fell rapidly and then continued to decrease at a slower rate.

Marrow kinetics are illustrated in figure 4 for a case of AML (case 4) and are similar to those previously reported [17] The principal feature is a sharp and early fall in large blast LI's accompanied by an equally early rise in medium blasts and the appearance of some labelled small cells. Peripheral blood LI values of the whole population were extremely low in the 1st h. Later a surge of large, medium and small blasts was observed (clear increase in LI values) with a maximum at the 48th h.



Fig. 1. Leukemic monocytes   and b and leukemic myeloblasts (c, d,) cultivated *in vitro*.   4 h after the begin of the culture the cytoplasm of the leukemic monocytes contains some vacuoles after 144 h large, vacuolized macrophages have developed (b Part of the cultured myeloblasts, (4, (d and   144 h of cultivation, contain distinct azurophilic granules d,   May-Grünwald-Giemsa (750

heparinized blood were employed which have been separated from the erythrocytes by 3 % gelatine Plasmagel, B. Braun, Melsungen The culture was carried out according to Hulliger et al. [16]. From   simultaneous cultures cells were gained daily by centrifugation with 700 g for 7 days.

Besides May-Grünwald-Giemsa stain the following cytochemical techniques were used nonspecific esterase substrate naphthol-AS-acetate) with and without addition of NaF 1.5 mg/ml [29], naphthylamidase [78], acid phosphatase [2], sudanblack-B stain [33] and methylgreen-pyronin stain [76]. Depending on the colour of the cytochemical reaction product nuclei were stained with acid Mayer hemalum or with Feulgen stain for DNS.

Results

Culture of leukemic monocytes. The *in vitro* cultivated leukemic monocytes of our 4 patients showed an enlargement as well as a considerable tendency to vacuolization [10, 23] fig. 1a, b) and phagocytosis.

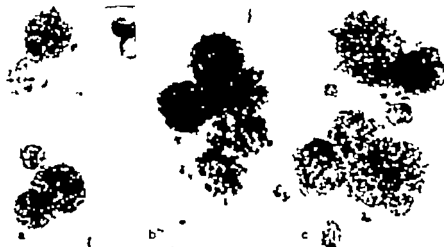


Fig 2. Leukemic monocytes cultivated *in vitro*. Naphthol-AS-acetate-esterase. (a) 24 h, 3 monocytes with marked, lymphocyte and neutrophil with only weak (few granules) esterase activity (b and) 144 h, esterase activity increased in the cultured leukemic monocytes (×750)

Morphologically these cells resembled the macrophages of *in vitro* cultures of normal monocytes [19 34] The mature neutrophile granulocytes degenerated during the first days [10] In some macrophages phagocytized cytoplasmic particels of neutrophile granulocytes were found. Besides lymphocytes which were not essentially different from those of the starting population, after 72 resp. 96 h only some (about 5‰) blast cells with heavy pyroninophilia were present in the culture The results of the cytochemical reactions in the cultured leukemic monocytes are summarized in table I

Nonspecific esterase In cases with relatively low activity of NaF sensitive nonspecific esterase in the monocytes this attribute remained during the whole cultivation. Indeed there was an increase of esterase activity (fig 3) but it was markedly weaker than compared with normal monocytes and the strongly positive cells of the cases 3 and 4

Acid phosphatase Twenty-four hours after the start of *in vitro* cultures the activity of the acid phosphatase in the leukemic monocytes was lower than in the corresponding cells of the peripheral blood. After 72 h and more marked after 144 resp. 168 h a strong increase of enzyme activity in the developed macrophages was detectable

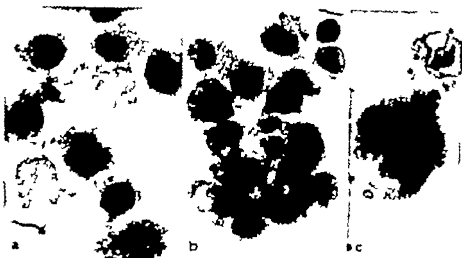


Fig 3. Acid phosphatase in leukemic monocytes cultivated *in vitro*. ( ) After 24 h distinct vacuoles are visible low activity of acid phosphatase. (b and) Strong acid phosphatase activity in macrophages after 144 h (and b ×500, c ×750)

(fig 3) This corresponds to the behaviour of cultivated normal monocytes. The cultures of the more immature less esterase containing leukemic monocytes showed besides strong positive macrophages, some cells with only little acid phosphatase activity

Naphthylamidase This enzyme reaction gave similar results as the reaction of acid phosphatase

Sudanblack B The sudanblack B stain in monocytes and the derived macrophages only slightly increased during *in vitro* culture Especially the wall of the small vacuoles were stained in macrophages.

Methylgreen-pyronin stain. The cytoplasmic pyroninophilia in the leukemic monocytes of the peripheral blood and at the onset of the culture was usually marked but it decreased during the course of the culture At the beginning of the culture the leukemic monocytes contained generally numerous and distinct nucleoli which were diminished in later periods. At this time some blast cells with a strong cytoplasmic pyroninophilia contained also conspicuous nucleoli They were similar to the blast cells occurring after antigenic stimulation of blood lymphocytes *in vitro* [9 10 16]

In vitro culture of leukemic myeloblasts Clearcut differences between cultured leukemic myeloblasts and leukemic monocytes were already

Table I. Cytochemical investigations on *in vitro* cultured leukemic monocytes

Time of cultivation	Case	Naphthol-AS-acetate-esterase	Naphthol-AS-esterase NaF	Acid phosphatase	Naphthylamidase	Sudanblack B	Pyroninophilia (methylgr pyronin)
1 day	1	+ − + − +	± − +	+	+	± − +	+ − + +
	2	+ − + − +	± − +	± − −	−	± − +	− − − +
	3	+ + − − − −	± − +	− − − −	+ − + +	± − −	+ − − −
	4	+ − − + + −	± − −	+ − − −	−	±	−
3 days	1	+ − − + + −	± − +	+ − + − −	− − + + +	− − − −	−
	2	+ − + − +	± − +	+ − − −	− − + −	+ − + +	+
	3	− − − + + +	± − −	+ − − + − −	− − − − −	− − + −	+
	4	+ + − + + +	± − +	+ + − − + −	+ + − + + +	+ − − +	−
6 days	1	− + − + − −	+	− − − + − −	− − + − −	−	+
	2	+ + − − − −	+	+ − − + −	− − + −	−	−
	3	+ + − + + +	± − +	+ − +	− − − + − +	− − + −	+
	4	+ + − − + +	+	− − +	− − − − − −	+	+

Positivity resp. enzyme activity ± uncertain, + weak, + + middle, + + + strong.
In most cells the activity increased compared to the first da
Only some + cells



Fig 4 In vitro culture of leukemic myeloblasts, 144 h. Naphthol-AS-acetate-esterase. ( ) Paramyeloblasts with only weak, (b) macrophage with strong esterase activity 800)

visible using May-Grünwald-Giemsa stain (fig 1) The vacuolization in the cytoplasm of myeloblasts was not as pronounced as in monocytes. Contrary to leukemic monocytes the main part of cultured leukemic myeloblasts contained an abundant azurophilic granulation

which was more marked than in the myeloblasts of the peripheral blood (fig 1d, e) During *in vitro* culture nuclear changes also occurred which simulated a nuclear maturation, but the cytoplasm continued to show a marked basophilia [23]

In the myeloblasts the high esterase activity typical for monocytes, was lacking (fig 4) The acid phosphatase activity increased after 72 h [1] During the course of the cultivation a part of the leukemic myeloblasts degenerated others were viable after 168 h and exhibited the above mentioned changes. After 86 resp 120 h besides myeloblasts few large macrophages with strong acid phosphatase and marked naphthol AS-esterase activity appeared which in their cytochemical pattern corresponded with the macrophages developed out of normal monocytes.

Discussion

The *in vitro* transformation of normal blood monocytes into macrophages was studied extensively by means of biochemical light and electronemicroscopic methods [3 6 7 8, 19 34] The increased content in hydrolytic lysosomal enzymes, which could partly be demonstrated with cytochemical techniques too [6 10] pointed to the functional changes within these cells [3 6 7] The simultaneously occurring increase in enzymes of the oxidative pathways can be interpreted as a sign of alteration in cellular metabolism [3 6 8] A similar transformation of monocytes occurred in the skin window experiment [28, 37] In one case of monocytic leukemia Nowell followed the behaviour of *in vitro* cultured leukemic cells and stated that, under given circumstances, leukemic monocytes change into typical large and vacuolized macrophages [23] Variing the incubation medium Oekkermann obtained spindle-shaped macrophages and giant cells [24]

In our cases we also found a marked morphological transformation of the leukemic monocytes associated with a strong vacuolization of the cytoplasm. The observed rise in acid phosphatase and naphthyl-amidase activity in the cultured leukemic monocytes parallels the biochemical and electronemicroscopical findings encountered in normal monocytes cultured *in vitro* [6, 8, 19 34] Both enzymes are thought to occur in lysosomes and in the Golgi apparatus [6, 22 28, 35 36] The slight increase in intracellular phospholipid content may

be due to the phagocytosis of degenerated neutrophiles and thrombocytes as well as to a *de novo* synthesis of lipids.

Comparing the results with cytochemical findings in cultured normal monocytes [10] only slight differences were detected. In 2 cases the leukemic monocytes in the peripheral blood and in the *in vitro* culture as well as the developing macrophages contained a somewhat decreased esterase activity. This was considered as further prove that leukemic monocytes in fact develop into macrophages.

These results confirmed that, under identical circumstances, leukemic monocytes show morphologic and enzymatic alterations, which agree with those of normal monocytes.

In cases with numerous immature cells in the peripheral blood, changes took place accordingly. Nevertheless very immature cells were not able to undergo a similar extensive transformation as mature cells. In some of these cells the morphological transformation did not correspond to the increase in hydrolytic enzymes.

The ability of leukemic monocytes to emigrate in the REBUCK skin window experiment during the first hours and to transform subsequently into large macrophages is of some diagnostic value for differentiating monocytic from acute myelocytic leukemia [27]. The *in vitro* culture of leukemic cells also shows differences in the behaviour of acute myelocytic and monocytic leukemia and is consequently applicable for differential diagnosis.

Summary

In vitro cultured leukemic monocytes transform into vacuolized macrophages. Cytochemical investigations (nonspecific esterase, acid phosphatase, naphthylamidase, sudan-black-B, methylgreen-pyronin) point to a marked increase of hydrolytic enzymes during macrophage transformation. The functional importance of these results is discussed. The examination of cell cultures with cytochemical techniques adds a further criterion for the differentiation of acute myelocytic and monocytic leukemias.

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Beta Thalassaemia Minor with an Unusually High Prevalence among Siblings

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Recently we described 21 cases of β-thalassaemia minor in two unrelated Swedish families. It was concluded that the abnormality might not be as uncommon in Scandinavia as is generally believed [1]

This view is supported by the finding of a third aboriginal Swedish family in which several members presented haematological signs of β-thalassaemia minor Most striking however was the high incidence of the abnormality in this family

Material and Methods

All the 23 living members of the family were subjected to thorough clinical and haematological investigation including haemoglobin electrophoresis. The pedigree is shown in figure 1 C 3 was the original patient. At the age of 19 years she was found to hav anaemia, and was subsequently treated with iron, both orally and without any effect.

Four persons in the pedigree were dead. A 1 died from renal failure at the age of 71, A 2 from heart failure t the age of 73, B 2 from pulmonary tuberculosis the ge of 22, and D 3 was stillborn.

The laboratory methods used were the same as described in previous publication [1].

Results

Clinical and haematological findings Of the 23 persons investigated 6 were completely normal clinically and haematologically The other 17 showed a raised Hb A_2 concentration and other signs of β-thalassaemia minor In these 17 patients the general examination did not reveal any relevant illnesses or abnormalities. Four of them, however suffered

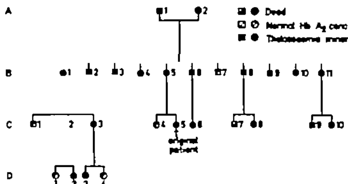


Fig 1

from arterial hypertension. The pertinent haematological findings are summarized in table I

Mild to moderate anaemia was found in 16 patients, and only C8 had a normal haemoglobin concentration. The red-cell count was low in 2 patients (C 10 and D 2) and high in 4 (B 3 B 6, C 6, and C 8) The mean red-cell volume was below normal in 11 patients. All patients showed varying degrees of anisocytosis, poikilocytosis, and polychromasia. Target cells were always found, and varied in number from 0 6 to 18.0 %. All the patients demonstrated ovalocytosis slight spherocytosis, and/or punctate basophilia. The MCH and MCHC were low in all patients. The serum iron and the total iron-binding capacity were normal in all 14 patients thus investigated. The reticulocyte count was raised in 13 patients (2.0–7.2) Intraerythrocytic inclusion bodies were not observed. The serum bilirubin concentration was not increased. The osmotic resistance of the red cells was determined in 2 patients (B 11 and C 5) and was increased in both. Coombs's direct and indirect tests were performed in C 5, and were negative In this patient the bone marrow showed increased erythropoiesis and 81 sideroblasts.

The mean Hb A concentration in the 17 patients was 4 4 (range 3 6–5 4) as compared to 2 0 % in the 6 healthy family members and 2.5 % (range 1 9–3.3) in 50 blood donors. The mean Hb F concentration was 1 7 % (range 0.2–4 4) as compared to 0 8 in the 6 healthy family members and 0 6 % (range 0.2–1.9) in the 50 blood donors. No abnormal haemoglobin components were found

Table I

Patient	Sex	Age years	Hb g/100 ml	Red cells mill/mm	MCV μ³	MCH γγ	MCHC %	Target cells %	Hb A_2 %	Hb F %	Serum iron μg/100 ml
B 1	F	65	9.9	4.5	76	22	29	2,0	4.1	1.7	107
B 3	M	62	12.6	5.8	78	22	28	2.1	5.1	1.9	51
B 4	F	60	10,9	4,9	77	22	29	2.0	4.0	2.1	86
B 5	F	48	9.9	4.7	78	21	27	8,8	4,3	0,3	113
B 6	M	55	11,8	5,7	79	21	27	4.1	5.1	1.7	60
B 8	M	42	10,6	4.9	76	22	29	2.1	4.3	2.8	121
B 9	M	40	11.4	3.6	72	20	28	7,8	4.1	3.1	–
B 10	F	48	10,0	4.4		23			4.0	0.5	–
B 11	F	45	10,7	4.1	79	21	30	2.2	4.2	1.1	108
C 2	M	36	10.5	4.9	77	22	28	4.8	4.1	3.1	119
C 3	F	27	9,3				23	18,0	4.1	1.9	103
C 5	F	22	10.8	4.3	81	24	26	2,1	4.4	0,4	115
C 6	F	19	11.4	5,0	75	20	27	5,0	4.3	1.7	116
C 8	F	11	11.8	5.3	80	22	28	1.2	4.8	1.4	–
C 9	M	23	11.1	4.2	97	26	27	17.0	3.6	0.2	96
C 10	F	20	10,8	3.9	97	27	28	2.0	3.8	0.2	83
D 2	F	6	9.0	3.7	81	21	29	0.6	4.4	4.4	102

Heredity From A 1 and A 2 the ancestors could be traced back for 4 generations, i.e. to the middle of the 18th century All of them were aboriginal Swedes, all having been born and having lived within the borders of the same small area of South-Eastern Sweden No consanguinity was found, and no relation to the two previously described Swedish β-thalassaemia families could be shown

Discussion

The haematological findings in these 17 patients strongly support the diagnosis of β-thalassaemia minor All had a raised Hb A_2 concentration, and most of them had slight to moderate hypochromic anaemia. In 5 patients an increased Hb F concentration was further evidence in support of the diagnosis. From the diagnostic point of view it is also of importance that no other causes of the haematological findings could be found above all, iron deficiency anaemia was excluded.

The only puzzling finding is the high incidence of the abnormality among the 10 living siblings in generation B. Since β thalassaemia is caused by a single autosomal incompletely dominant gene a disease incidence of 50 % or less may be expected if one of the parents, A 1 or A 2 was a carrier of this gene. Instead, an incidence of 90 % was found The probability that the great difference between the expected and the found incidence is due to chance is indeed very small, but it seems to be the only explanation that fits in with the facts. If both parents, A 1 and A 2, were heterozygous for the β-thalassaemia gene 50 % of their children could be expected to be heterozygous (β-thalassaemia minor) 25 % homozygous (β-thalassaemia major) and 25 % normal. Since most patients with β-thalassaemia major die young it is very likely that most (60–70 %) grown up siblings have β-thalassaemia minor while no major case is found In this family however such an explanation can be excluded because A 1 and A 2 had no children other than those reported in the pedigree, and none of them showed any symptoms or signs characteristic of β-thalassaemia major

Summary

In an aboriginal Swedish family consisting of 23 living members 17 presented unequivocal signs of β-thalassaemia minor The high disease incidence (90 % among 10 siblings) was

a remarkable feature not characteristic of β-thalassaemia minor. In the absence of family members with suspected or proved β-thalassaemia major it was concluded that the high incidence was due to chance.

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An *in vitro* Study of the Effect of Arsenic (As_2O_3) on Blood Clotting

T P Elias

The effect of drugs studied by means of modern techniques may reveal many facts which are hitherto overlooked. An *in vitro* study of arsenic (As_2O_3) a remedy widely used at one time is made here, so as to find its effect on the blood clotting system mainly by the help of thrombelastograph.

Experimental

The reagents and chemicals used were that of E. Merck AG Darmstadt (Analytical Quality) clotting factors that of Behringwerke AG Marburg/Lahn. Blood was obtained from healthy persons between 15 and 45 years of age by venous puncture in plastic syringes an aqueous solution of 3.2% trisodium citrate in proportion 1 9 being used as the anti-coagulant. Arsenic (As_2O_3) 1 g in about 100 ml of double distilled water was dissolved by boiling for about 30 min. After filtration the quantity of dissolved As_2O_3 was assayed according to B. P [2] and the volume was made up, to contain 1% of As_2O_3. Further dilutions were carried out in double distilled water according to necessity For control experiments 0.9% NaCl (physiological saline) was used unless otherwise stated.

The estimation of blood clotting factors were done according to Schwick and Storiko [11].

Isolation and washing of platelets were done according to Spaet and Zucker [13] from citrated normal human blood and suspended in NaCl 0.9%.

The platelets spreading capacity was done according to the method of [illegible] and Busch [1].

Hellige Thrombelastograph by Fritz Hellige & Co. was used for the coagulation studies. 0.3 ml of plasma or fibrinogen solution was made t clot in the cuvettes of the instrument by the addition of $CaCl_2$ solution or thrombin solution so as to maintain always constant volume of 0.36 ml [6].

Dedicated to Prof. Dr W H. Hauss on the occasion of his 60th birthday

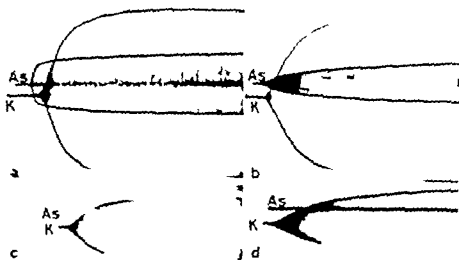


Fig 1 Effect of As_2O_3 on normal blood and plasma clots. K = control, As = arsenic trioxide a. Arsenic in plasma clotted by recalcification. b. Arsenic whole blood clotted by recalcification. c. Arsenic plasma clotted by thrombin. d. Arsenic whole blood clotted by thrombin. 0.25 ml plasma or citrated blood mixed with 0.05 ml NaCl 0.9% or arsenic [illegible] 0.05 ml clotted by means of 0.06 ml aqueous $CaCl_2$ 1.29‰ or 0.06 ml *thrombin* 10 NIH units (test-thrombin RIFT 18, Behringwerke AG diluted with NaCl 0.9%)

Results

It can be seen from figure 1 that the effect of arsenic so as to reduce the me (maximum thrombus elasticity) is greater in the whole blood than in the plasma Further it can be noted that the reaction time r of arsenic is about ½ lesser than that of the control (fig 1a and b) In figure 1c and d where thrombin is used for coagulation and the citrate remains in the blood or plasma without being neutralized with $CaCl_2$, the elasticity modulus has not increased as in the *earlier* figures, showing the necessity of enough calcium ions for a normal clot On the other hand, from *figure* 2a it is quite clear that when *clotting* is done with enough fibrinogen by means *of thrombin* arsenic has got an opposite effect of making me *of the* clot a little more *than* the control (fig 2a As) This effect in conditions of *varying* concentrations *of* fibrinogen thrombin and arsenic *can* be *noted* in figure *3* The maximum elasticity depends upon an increased arsenic concentration decreased fibrinogen concentration and an optimum thrombin concentration ranging between 10 to 20 *NIH* units.

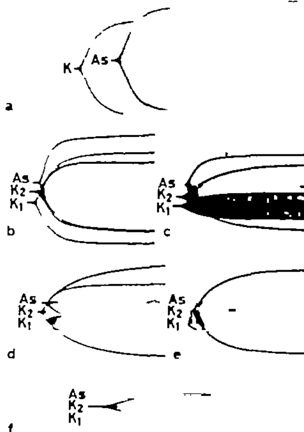


Fig 2. Thrombelastogram of fibrinogen clotted with thrombin in the presence of arsenic and washed platelets. a. Fibrinogen solution (40 mg/ml) 0.25 ml mixed with 0.05 ml NaCl 0.9% in control K and 0.05 ml arsenic 1% in As. b–f Fibrinogen solutions b = 40 mg/ml c = 20 mg/ml, d = 10 mg/ml, e = 5 mg/ml, f = 2.5 mg/ml. K_1 0.1 ml NaCl 0.9%; K_2 + 0.05 ml NaCl 0.9% + 0.05 ml platelets 1,000,000/mm³ As + 0.05 ml arsenic 1% + 0.05 ml platelets 1,000,000/mm³ Fibrinogen solution Human fibrinogen, Konzentrat, Behringwerke AG dissolved in NaCl 0.9%. Experimental conditions as in figure 1. In e and f another sample of fibrinogen solution was used.

Further from figure 2 it can be seen that when the amount of fibrinogen is sufficient for a strong clot, the presence of an extra amount of platelets has no further effect (fig 2b K_2) even though arsenic still retains its property of increasing the me in the presence of platelets (fig 2b As) But when the amount of fibrinogen is reduced

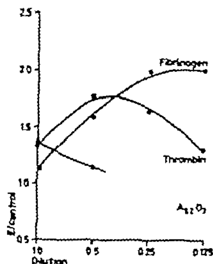


Fig 3 Elasticity of fibrinogen clot with varying concentrations of thrombin, As_2O_3, and fibrinogen. Experimental conditions as in figure 2a.

and the amount of platelets is kept constant, it can be seen that the control K_1 is reduced progressively (fig 2b c, d e and f) But K_p, the control in presence of platelets, retains its original maximum elasticity even at the cost of progressively reduced fibrinogen except in figure 2f where the fibrinogen amount is reduced to the maximum. Even in figure 2f K_4 is comparatively much bigger than K But the presence of arsenic destroys this property of maintaining the maximum elasticity by platelets in proportion to the decrease of fibrinogen (as in fig 2b c d e and f)

Arsenic increases factor V and reduces the recalcification time but has not any effect on other factors (table I)

Experiments done to know in detail the action of arsenic on plasma showed the following facts. The time of incubation of arsenic with the plasma is not very important (table II) The slight acid pH of arsenic is not responsible for its effect Arsenic 1 neutralized by NaOH so as to have a pH 7.2 (read in pH/m) shows the same effect if not more Other materials of acid pH like boric acid and potassium dihydrogen phosphate have not the same effect (table II) A buffer was purposely avoided as it was thought to have a study of arsenic in the pure form without the interference of any foreign substance and also the in-



Fig 4 The spreading capacity of platelets in presence of As_2O_3. Control, normal plasma. b 0.25 ml plasma + 0.05 ml arsenic 1%. 0.25 ml plasma + 0.05 ml arsenic 0.1%. d 0.25 ml plasma + 0.05 ml arsenic 0.01%. Under oil immersion the following variety of platelets are seen Giant type: nil; big sized. nil small sized 30%. transitional type: 36%. spider type 34 %; b Only some very few distorted spider type cells were seen; Very few cells consisting of 5 % transitional type and 95 % spider type: d Small type: 3%; transitional type: 19%; spider type: 78%; the total number comparatively less.

clusion of the buffer would reduce the actual amount of plasma when it is difficult to get an optimum clot in the thrombelastograph.

An attempt to study the effect of arsenic on isolated and washed blood cells showed that arsenic acts only on platelets and it has no action on erythrocytes or leukocytes as far as the clotting of blood *in vitro* is concerned. Table III shows that the addition of platelets increases the me of high centrifuged plasma. But by the addition of arsenic the activity of platelets is almost completely destroyed, depending to certain extent upon the number of platelets. Also the reaction time r is reduced in every case after the addition of arsenic. The effect of arsenic on dried platelets is negligible in this respect (Exp II in table III) It is not possible to measure k in presence of arsenic, in most cases as the elasticity does not reach a thickness of 20 mm.

Table I

Experiment	Control	Arsenic
1 Factor V	38 sec (100%)	18 sec (more than 200%)
2 Factor VII	37 sec (92%)	36 sec (95)
3 Factor VIII	15 sec (60%)	16 sec (56%)
4 Factor IX	17 sec (54%)	17 sec (54%)
5. Quick test	16 sec (55%)	16 sec (53%)
6. Recalcification time	90 sec	52 sec

Five ml of citrated plasma or serum were mixed with 1 ml of 1% arsenic and the experiments were done In control 0.9% NaCl was used instead of arsenic

Table II

Experiment		sec	k, sec	me
I	1 Control incubated 5 min	520	150	2 7
	2 Arsenic 1	180	300	32
	3. Control incubated 30 min	270	90	237
	4 Arsenic 1%	120	300	32
II	1 Control	360	150	170
	2. Arsenic 1% pH 6	193	750	31
	3. Arsenic 1% pH 7.2	19.	900	[illegible]
	4 Pot dihydrogen phosphate 1 pH 5	300	120	233
	5. Boric acid 1 pH 6.6	240	90	203

Experimental conditions as in figure 1

The spreading capacity of platelets studied shows that in presence of arsenic the platelets are destroyed or distorted and washed away in the process of staining depending upon the amount of arsenic (fig 4)

In figure 5 it can be seen that by progressive dilution the effect of arsenic is nullified at a dilution of 10 * after which there is an opposite effect of increasing the elasticity at a dilution of 10 Further dilution reduces this effect The dilutions were carried in double distilled water and a control of double distilled water was used instead of NaCl 0.9 %. The effect was conspicuous when very fresh blood was used.

Table III

Experiment	Plasma used	Additions	sec	k, sec	ma modulli
I)	High centrifuged	NaCl 0.9%	285	300	58
b)	High centrifuged	NaCl + arsenic 1%	260	–	13
)	High centrifuged	NaCl + platelets 1000 000/mm³	240	180	117
d)	High centrifuged	ars. 1% + pl. 1000,000/mm³	210	–	18
)	High centrifuged	NaCl + pl. 500,000/mm³	230	150	83
f)	High centrifuged	ars. 1% + pl. 500,000/mm³	165		17
g)	High centrifuged	NaCl + pl. 250,000/mm³	225	270	74
h)	High centrifuged	ars. 1% + pl. 250,000/mm³	150	–	16.5
i)	High centrifuged	NaCl + pl. 125,000/mm³	210	285	60
j)	High centrifuged	ars. 1% + pl. 125,000/mm³	133	–	15.5
II.)	High centrifuged	NaCl 0.9%	510	300	57
b)	High centrifuged	NaCl + ars. 1%	490	1,260	32
)	High centrifuged	NaCl + dried platelets	480	270	47
d)	High centrifuged	ars. 1% + dried platelets	360	330	35
III.)	High centrifuged	NaCl 0.9%	450	510	43
b)	High centrifuged	NaCl + ars. 1%	420	–	18
)	High centrifuged	NaCl + erythrocytes 0.05 ml	390	420	43
d)	High centrifuged	ars. 1% + erythrocytes 0.05 ml	390	–	20
)	High centrifuged	NaCl + leukocytes	420	450	42
f)	High centrifuged	ars. 1% + leukocytes	390		19

Citrated plasma was centrifuged t 3,000 rpm for 30 min. 0.2 ml were mixed with 0.1 ml of NaCl 0.9% for control, or 0.05 ml arsenic 1% and 0.05 ml NaCl 0.9%, or 0.05 ml of platelet suspensions of required strength (the number of platelets being adjusted with NaCl 0.9% used as diluting agent) with 0.05 ml NaCl 0.9%, or ars. 1% 0.05 ml were used in the first experiment. In experiment II, human dried platelets from Behringwerke AG, Marburg/Lahn, suspension of 5 mg in 2 ml 0.9% NaCl was used. Leukocytes isolated from the blood of leukaemic patient by differential centrifugation and washed several times in physiological saline, 0.1 ml of the packed cells suspended in 0.5 ml 0.9% NaCl, and erythrocytes prepared from normal blood in the same manner as bov was used in experiment III in quantities similar to that of platelets in experiment I.

Discussion

An *in vitro* preliminary study of the action of arsenic (As_2O_3) is attempted to know its specific effect on blood clotting as previous studies seem to be quite accidental, observed either in arsenic poisoning or an overdose of arsenic containing organic chemicals in patients

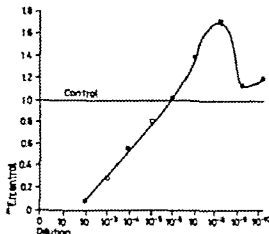


Fig 5. The elasticity of whole blood clot in presence of arying dilutions of As_2O_3. Double distilled water used at the place of arsenic in the control. Experimental conditions in figure 1b.

[3 4 8, 9 10 14 16] It is interesting to note that a normal elastic clot is formed either by a large amount of fibrinogen alone (more than ten times the amount found in normal blood) or a less amount of fibrinogen in combination with platelets, or plasma or blood containing normal amount of fibrinogen and enough platelets and Ca Deficiency of any of these three factors mainly fibrinogen platelets or Ca will tell upon the normal elasticity of the clot Thus observation is in agreement with that of WEINER and WEINBERG [15] and SOULIER *et al.* [12]

Arsenic seems to act upon the platelets, first favouring the viscous metamorphosis releasing more phospholipids and stimulating factor V (factor V is the last factor in the prothrombin conversion to thrombin [7]) and thereby activitating thromboplastin favouring the rapid formation of thrombin when blood or plasma begins to clot more rapid than a control But as arsenic acts further probably reacting with the SH groups of the platelet contents, the cells are completely destroyed as a result of which its normal function of forming an elastic clot in combination with fibrinogen and Ca is no more present resulting in a weak clot Even though arsenic favours probably the polymerization of fibrin monomer forming a stronger clot when only fibrinogen and thrombin are present) in normal blood in opposite

effect is observed. This phenomenon can be explained as follows: when the amount of fibrinogen is decreased progressively the elasticity is also proportionately reduced, and with amounts of fibrinogen similar to that in normal blood the elasticity of control is practically negligible. But the presence of sufficient platelets in combination with very small amount of fibrinogen makes the maximum elasticity (me) sufficiently strong as to appear like that of normal blood or plasma or even a very high concentration of fibrinogen. But when the platelets are destroyed by arsenic the elasticity is also reduced leaving behind the very small effect of the remaining fibrinogen only. The extreme importance of platelets in comparison to fibrinogen and Ca^{++} to form a strong clot in normal blood is clear from these observations. The role of fibrinogen seems to be to form only a basic net work over which really the platelets build the strong clot. The observations in fig. 5 confirm the idea of blood coagulation as an active living process in which fresh and active platelets resist the action of arsenic.

It can be concluded that arsenic has definite action on platelets and in coagulation of blood is not at all related to its well known action on leucocytes [5] and also that it has practically no action on erythrocytes.

Acknowledgement

The author thanks Prof. Dr. W. H. Hauss, Director of Medizinische Klinik and Poliklinik, University of Münster for providing all the facilities for the work and Dr. med. Thomas Bechtold for the keen interest and suggestions made in the work.

Summary

The action of arsenic (As_2O_3) on blood coagulation is studied mainly by thrombelastography.

Arsenic is found to act on blood platelets probably destroying them and reducing the elasticity of the blood clot, even though it appears to favour the clot formation by releasing the platelet phospholipids at the beginning. Arsenic favours the fibrinogen-fibrin conversion in the presence of thrombin, but this does not affect much its action on normal blood clot.

In very great dilution, as 10^{-9} can produce even an opposite effect of increasing the elasticity of the blood clot.

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Glycogen Content of Leukocytes of Some Animal Species

N. C. JAIN

Very little is known about the glycogen content of leukocytes of animal species [1]. Therefore the distribution of glycogen in the formed elements of blood of some animal species was studied by the periodic acid-Schiff (PAS) technique.

Material and Methods

Blood samples (2 to 5 ml), using disodium ethylene diamine tetraacetate anticoagulant (2 mg/ml of blood), were obtained from 59 dogs, 46 cats, 8 horses, 15 cows, 2 one-week-old twin calves, 6 sheep, 6 goats, 18 rabbits, 21 guinea pigs, 20 rats, and 3 monkeys. All animals were apparently clinically healthy.

Air-dried coverslip blood smears were stained 2 to 3 days after preparation [1]. The smears were fixed in methanol [1] and processed essentially following the PAS technic outlined by HAYHOE *et al.* [6]. The Schiff' reagent, prepared according to the method of de TOMASI [8], and the periodic acid solution were stored at 4 °C, allowed to attain room temperature before staining, and used only once. Control smears were exposed to filtered fresh human saliva for 30 min or to 1% aqueous malt diastase[1] for 60 min at 37 °C, and stained similarly.

The PAS reactivity of the leukocytes was graded from 0 to 4 on the basis of the intensity and extent of staining. A preliminary comparative study on influence of the anticoagulant on PAS staining revealed no significant differences in the reactivity of neutrophils of the dog, cat, cow, horse, and sheep when either EDTA or heparin was used. Blood samples from 2 animals of each of the species were used for this purpose.

Results

The PAS reactivity of leukocytes varied among different species (table I). Neutrophils invariably stained strongly, whereas lymphocytes, monocytes, and eosinophils were either negative or weakly posi

[1] Alpha-amylase (Diastase), Type V-A, from Malt, Sigma Chemical Co., St. Louis, M

Table I. Distribution of glycogen in the formed elements of blood of some animal species

Animals	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Erythrocytes	Thrombocytes
Dog	0–4	0–t	0–1	0–t	0	1
Cat	1–4	0	0	0–t	0	1
Cow	0–4	0–t	0–1	0–1	0	1
Horse	0–4	0–1	0–t	0–1	0	1
Sheep	*1–4*	*0–2*	*0–1*	*0–1*	*0*	*1*
Goat	1–4	0	0–1	0–1	0	1
Monkey	0–3	0	0–3	2	0	1
Guinea pig	1–4	0	0	0–1	0	1
Rabbit	0–3	0	0	0–1	0	1
Rat	0–3	0	0	0	0	1

0–4 represents different grades of PAS staining. 0 = negative; t = trace; 1 = slight; 2 = moderate; 3 = marked; and 4 = deep and intense PAS reaction.

Leukocytes of the twin calves had similar glycogen distribution. In contrast, a few erythrocytes of the calves were PAS-positive. The proportion of PAS-positive erythrocytes in such smears was similar to that of reticulocytes seen in duplicate smears stained with the new methylene blue stain [9].

tive. Platelets had small amount of PAS-positive material. Erythrocytes were PAS-negative; however, in 2 young twin-calves a few erythrocytes were distinctly PAS-positive. In control smears treated with saliva or malt diastase, except for a few neutrophils and eosinophils, which had only a trace of pink cytoplasmic staining, all leukocytes and platelets were PAS-negative. The staining intensity of PAS-positive calf erythrocytes was only partially reduced.

Neutrophils of all the species generally contained distinct or indistinct purplish red granules which were usually similar in size and staining characteristics, evenly distributed in the cytoplasm, and smaller than or comparable to the specific neutrophilic granules. In addition, a small proportion of neutrophils had a few large (up to 2 μm) deep red granules (fig. 1 and 2). The PAS reactivity of neutrophils varied from species to species and also among animals of the same species. The staining intensity of mature neutrophils did not seem to vary with their nuclear lobulations. It seemed that the overall glycogen content of neutrophils of the dog, cat, and rabbit was similar but comparatively greater than that of the neutrophils of other animals.

The PAS-positive lymphocytes contained one or a few small deep purplish-red granules, or had diffuse purplish-red staining areas in the

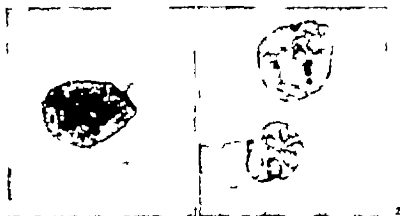


Fig 1 2. Peripheral blood smears stained by the periodic acid-Schiff (PAS) technique. A feline (fig 1) and 2 ovine (fig 2) neutrophils containing large PAS-positive granules (1,500)

cytoplasm. The monocytes were usually PAS-negative, but in some species an occasional monocyte had either pink staining cytoplasm (dog and cat) or a few PAS-positive granules (cow and monkey)

The specific granules of eosinophils were PAS-negative. In some species a variable number of eosinophils had slight pink staining material in the intergranular cytoplasm. This was quite distinct in the equine eosinophil. In the dog cat, and sheep some eosinophils had a few refractile red granules.

Platelets were faintly stained or had distinct fine to large deep red granules which sometimes coalesced to form large clumps. Some platelets had both fine and large granules this was quite prominent in the cat and the dog

Discussion

The PAS staining technic, the most common method to demonstrate glycogen gives positive reaction with a number of substances the presence of glycogen is recognized only when control preparations exposed to α-amylase or saliva before staining show negative or very little reaction. The efficiency of the enzymatic removal of PAS-positive material depends on the fixative used [4] It was shown that only acid-soluble glycogen (lyoglycogen) is demonstrated by the PAS technique [12]

Glycogen is present in the cytoplasm of neutrophils of man horse dog cat and cow the cells of the first 3 species are strongly PAS-positive and of the other 2 only slightly positive [1] The PAS-positive material in normal human neutrophils produces an irregular stippling of the cytoplasm [5] and consists almost entirely of glycogen [4] In equine neutrophils, a diffuse cytoplasmic staining as well as numerous granules of different size may be seen [10]

The staining intensity of cells of the granulocytic series increases with maturation the myeloblasts being PAS-negative and mature neutrophils being strongly positive [4] The PAS reactivity of mature neutrophils in the peripheral blood also varies In the present study varying amounts of glycogen were found in almost all neutrophils in the peripheral blood of all the species examined The glycogen content of mature neutrophils did not appear to increase with shift to the right.

Both in man and in animals, glycogen may be present in neutrophils in one or more forms or sites including the specific granules, whereas in the eosinophils it seems to have only cytoplasmic distribution. The presence of large glycogen droplets in a small number of neutrophils (fig 1 and 2) may either constitute another site of storage or indicate enlargement of small granules due to excessive deposit or metabolic derangement Glycogen was demonstrated as a constituent of the specific granules or only as a cytoplasmic component when the PAS technique was used in combination with other methods [2 3] Distinct small dense glycogen granules are seen in neutrophils in the electron microscope [11] Recent studies with homogenized human [7] and equine [10] leukocytes have shown that glycogen is present in varying amounts both in the granular fraction and in the microsomal fraction In the study with human leukocytes one or several nonglycogenic substances were also found and it was considered that the nonglycogenic substances are quantitatively more important for the PAS reaction than the glycogen

The finding of a few PAS-positive erythrocytes in young calves was unexpected This reaction appeared to be due both to glycogen and nonglycogenic PAS-positive substance(s) since treatment with malt diastase did not abolish the PAS reactivity of such erythrocytes. Although the distribution of PAS-positive erythrocytes was similar to that of reticulocytes seen in duplicate blood smears stained with new methylene blue the small number of reticulocytes normally present in the peripheral blood of some mature dogs and cats were

PAS-negative. It remains to be determined whether this is a species characteristic or a peculiarity of newborns.

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Summary

The distribution of glycogen in the formed elements of blood of the dog, cat, horse, cow sheep, goat, rabbit, guinea pig, rat, and monkey was determined using the periodic acid-Schiff (PAS) technique. Neutrophils of all the species had largest amount of glycogen, whereas lymphocytes, monocytes, and eosinophils had either no demonstrable glycogen or very small amounts. Platelets had small amount of glycogen. A few PAS-positive erythrocytes were seen in 2 one-week-old calves only

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14. Kongress der Deutschen Gesellschaft für Hämatologie

11 13. September 1969 in Kiel

Vorläufiges Programm. Generalthema: Die Milz und ihre Erkrankungen. 1 Morphologische Grundlagen. 2 Funktionelle Grundlagen. 3 Die einzelnen Blutzellsysteme in ihrer *Beziehung zur Milzfunktion und zu den Milzkrankheiten*) *Milz und Erythrozyten, b)* Milz und Thrombozyten, c) Milz und Granulozyten. 4 Hypersplenismus. 5. Tumormetastasen in der Milz. 6. Rundtischgespräch Gibt es eine splenopathische Markhemmung und einen Morbus Banti

Kongresspräsident Prof. K. La rar Anmeldung und Anfragen Dr D. Harms, Pathologisches Institut der Universität, Hospitalstrasse 42, *D-23 Kiel* (Deutschland)

International Symposium on Blood and Tissue Antigens

Ann Arbor Michigan, September 17 19 1969

The immediate objectives of this meeting are ( ) to present some of the latest developments in blood and tissue antigens (b) to promote an activ dialogue between immunogeneticists and biochemists ( ) to provide a forum for stimulating speculative discussions. Topics under discussion will include various aspects of immunogenetics, cellular blood group *and histocompatibility antigens, structural studies of the water soluble glycoprotein an igens,* their specific enzymatic degradation and biosynthesis. Sponsored by the Glycoamino Glycan Glycoprotein Group of biochemists, the meeting will honor the retirement of *Prof* W T J Morgan, (Great Britain)

Further informa tion may be obtained from D vid Aminoff Ph. D Chairman, Simpson Memorial Institute University of Michigan, *Ann Arbor* Michigan 48104

VIth International Tissue Research Conference

The VIth I ternational Tissue Conference entitled *Blood Cell as Tissue* will be held t the Lankenau Hospital, Philadelphia, Pa., on October 30 31 1969 The following topics will be discussed Regulatory mechanisms, metabolism and function of normal nd b normal cells, and recent developments in therapy For further information please contact William L. Holmes, Ph. D., Di ision of Research, Lankenau Hospital, Lancaster and C ty Line A enues, *Philadelphia Pa. 19151* (USA)

International Committee for Standardization in Haematology

The I ernational Haemoglobincyanide Reference Solution, which is prepared by the Rijks Instituut oor de Volksgezondheid, Utrech Netherland) under the auspices of he I ternational Commi tee for Standardization in Haematology (ICSH) and issued sinc May 1965 as project sponsored by the Council of Europe has recently been established by World Health Organiza ion as the I ernational Haemoglobincyanide Reference Preparation It is ailable free of charge on request to national laboratories working in haema logy or to interested ind idual workers and is intended for hecking the purity and content of haemoglobincyanid reference solutions to be used in the daily practice of haemoglobinometry I consists of an aqueous haemoglobincyanide sol tion equivalen to haemoglobin onten of pproximately 60 mg 100 ml, dispensed in 10 ml mpoules and packed in boxes of ten. I is tested and regularly hecked by number of control laboratories nominated by ICSH

Further information ma be obtained from Dr A. H. Holtz secret ry ICSH E pert Panel on Haemoglobinometry R jks Instituut oor de Volksgezondheid, Utrech Netherland

Brit J Haema Suppl *13* 71 196

Wld Hlth Org techn Rep Ser *384* (1968)

Acta haemat. 41: 257–263 (1969)

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Peripheral Nerve Function in Pernicious Anemia before and after Treatment[1]

D. Lockner, P. Reizenstein, A. Wennberg and L. Widén

Treatment of pernicious anemia with cyanocobalamin causes a remission which is incomplete hematologically [13] and neurologically. As early as the 1870's it was known that symptoms of nervous system disease may occur in patients with pernicious anemia [7, 8]. The frequency of neurological symptoms, mostly in the legs, varies greatly in different series. In Björkenheim's survey [2] the lowest figure is 17 % [6] and the highest 97.7 % [1]. Myelopathy is the form of neurological damage most frequently mentioned.

The object of the present investigation was to study the extent and the response to treatment of peripheral neuron damage (neuropathy) for which sensitive objective techniques – electroneurography (ENeG) – have become available. The frequency of objective changes in the absence of subjective symptoms will be examined and pathogenetic possibilities will be discussed.

Case Material

Extent of neuropathy. Twenty-seven patients (12 men and 15 women), aged 35 to 80 years (mean 65.3), with pernicious anemia in relapse or remission were studied. The diagnosis was confirmed by determination of the vitamin B_{12} concentration in serum (borderline value = 150 pg/ml) [11] and the Schilling test (borderline value ≥ 11 % of the administered quantity of vitamin B_{12} excreted during the first 48 h [12]). Fourteen of the patients had no neurological symptoms. Six patients had sensory symptoms only and 7 patients had sensory

[1] This work was aided by grants from Ekhaga Foundation, Stiftelsen Gustaf and Tyra Svenssons Minne and Loo and Hans Ostermans fond. A preliminary report was published in 1964.

symptoms and loss of the ankle jerks. The sensory symptoms consisted of diminution or loss of vibration and joint position sense, in some cases also impaired tactile sensibility in the legs, in 2 patients in the hands as well. Five patients were untreated at the time of examination 4 of them had neurological symptoms. The remaining patients had received vitamin B_{12} treatment for varying periods up to 12 years.

Response to treatment. A further 7 patients (3 men and 4 women) with untreated pernicious anemia as defined above were studied. Ages were between 53 and 83 years (mean 69.3 Two of these patients were examined twice, the remaining 3–5 times each.

Controls. 117 subjects aged 7 to 79 years (mean 47.5) were examined. They were either healthy individuals or patients referred for routine examination on account of unilateral nerve damage, usually traumatic. The serum vitamin B_{12} concentration was determined in about half of the controls over the age of 60, to exclude the presence of pernicious anemia.

Methods

To study objective signs even in the absence of subjective symptoms, the median nerve was examined in each case electroneurographically [3 4, 5 15]. Subjective symptoms are rarely found in the arms. The position of the electrodes is shown in figure 1 The conduction velocity of the motor fibres was determined and the action potentials of the median nerve recorded. The nerve action potentials were recorded, using a unipolar technique, with needle electrode, electrolytically polished to tip diameter of 5–15 μm, inserted through the skin. The indifferent recording electrode consisted of a chlorided silver disc. T insert the needle as close as possible to the nerve, a standardized procedure was used with the recording needle as stimulating cathode [3].

Results

Extent of neuropathy The mean amplitude of the nerve action potential was 84 6±8.33 μV in the 27 patients with pernicious anemia and 193±8.60 μV in the 117 controls. This difference is highly significant.

In the controls a highly significant correlation was found between age and the amplitude of the nerve action potential, the coefficient of correlation being −0.66. No such correlation was found in the patients (coefficient of correlation −0.22) The difference between the coefficients of correlation in the patients and the controls is highly significant ($p < 0.001$)

Figure 2 shows the amplitudes of the nerve action potentials in the patients with pernicious anemia plotted against age In 9 patients (33) the amplitude is outside the normal limits, indicating electroneurographical evidence of neuropathy In a further patient who had a normal value at the first examination a second examination 7 months later revealed a pathological value despite vitamin B_{12} treatment.

Neurological symptoms were present in 7 (78) of the 9 patients

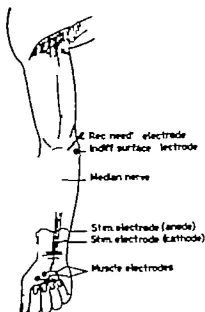


Fig 1 Position of electrodes for recording of action potentials in the median nerve and thenar muscles. The nerve was stimulated through surface electrodes at the wrist. The train of impulses elicited in this manner gave rise to ( ) nerve action potential recorded with needle electrode in the cubital fossa and (b) twitch in the thenar muscles, whose action potential was recorded with surface electrodes.

with an abnormal ENeG but only in 6 (33 %) of the 18 patients with a normal ENeG The difference is significant at the 95 % level ($p < 0.05$)

Response to treatment. Twelve patients were untreated at the time of examination, 4 of them had electroneurographical signs of neuropathy Seven of the patients were followed up with repeated examinations. The amplitude of the nerve action potentials in 5 patients examined more than twice during the course of treatment varied between a decrease of 0.089 % daily and an increase of 0.036 % per day Thus the change in any patient was not significantly distinguishable from zero Neither was the weighted mean change (a decrease of 0.025 % ± 0.030 % per day) significantly distinguishable from zero, nor was any improvement noticeable in the 2 patients examined only twice, who had potentials of 73 and 76 µV before and 68 and 89 µV between 5 and 10 months after the commencement of treatment, respectively

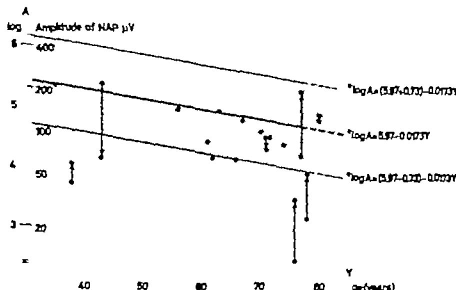


Fig. 2. Regression function (heavy line) of the correlation between amplitude of the nerve action potential (NAP) and age in the control series. The mean ± 2 × the residual variance is indicated by the shaded area. Points denote the amplitude of the nerve action potential in the patients with pernicious anemia. Patients examined twice are represented by two points joined by an arrow the arrow-head marking the value at the second examination.

Discussion

Controls. The present investigation shows that in healthy subjects there is a negative correlation between the amplitude of the nerve action potential and age. This observation is in agreement with that of Buchthal and Rosenfalck [3] They divided their series into 3 age groups 18–25 40–61 and 70–88 years, and noted different values for the amplitude in the 3 groups. A regression analysis of the amplitude with respect to age for the whole of the present material showed that a decrease in amplitude occurs at an early age, probably even before the age of 20 Moreover the reduction in amplitude in absolute figures seems to be accentuated at lower ages, since a logarithmic function fits the material better than a linear

It would have been desirable to examine also the nerves of the lower limbs. The reason this was not done is that this latter procedure causes the subjects far greater discomfort, owing to the necessity of using a summation technique with average response computer

Extent of neuropathy. In the arms where only 7 % of the patients had subjective symptoms, almost 5 times as many had objective signs of neuropathy. Experience shows that in mild diffuse neuropathies, electroneurographic as well as clinical signs of disturbance of nerve function usually appear earlier or are more pronounced, in the lower limbs. If the factor 5 could be applied to the legs also, where almost half of the patients had subjective symptoms, all would have had objective signs of neuropathy.

Even among the earliest cases electroneurographical signs of neuropathy were found. These findings suggest that peripheral neuron damage (neuropathy) is not exceptional in pernicious anemia and that neuropathy is not a late complication – rather it seems to be an early intrinsic part of the disease.

Six of the patients with pernicious anemia had normal electroneurograms in the arms despite symptoms in the legs. In our experience, it is uncommon for patients with sensory symptoms in the lower limbs due to diffuse neuropathy to have a normal electroneurogram in the arms, even when these are symptom free. Consequently it is possible that in one or more of the 6 patients the symptoms were due, not to neuropathy but to mild myelopathy. This most often begins in the posterior columns of the mid-thoracic cord [10] and in the early stage may give rise to sensory disturbances that are indistinguishable clinically from peripheral neuropathy. A normal electroneurogram in a patient with pernicious anemia and sensory symptoms would therefore seem to give reason to suspect myelopathy [9].

Since our preliminary communication [14] MAYER [9] has published a report on the peripheral nerve function in patients with vitamin B_{12} deficiency. His results differ from ours in that he found no change in peripheral nerve function of the median nerve in a) healthy subjects under the age of 80 years, b) patients with vitamin B_{12} deficiency without neurological symptoms, and c) patients treated with vitamin B_{12} for more than one month. The differences between the results of the two investigations are probably due to the fact that MAYER studied primarily the conduction velocity of the nerve which, in our experience [15] is a less sensitive index of peripheral nerve function than the amplitude and shape of the nerve action potential.

The low amplitude of the action potentials seems to indicate impaired transmission of impulses in the peripheral nerves. This does not necessarily give rise to clinical symptoms. Loss of a few nerve fibres

and/or increased dispersion of conduction velocities in different fibres can presumably occur without the appearance of sensory disturbances.

Response to treatment There was no significant response at all, but neither did any deterioration occur during treatment. No reason is known for the lack of effect of even early treatment in mild cases.

Summary

The peripheral nerve function was studied by electroneurographical examination of the median nerve in 34 patients with pernicious anemia and 117 controls. In the controls, negative correlation was found between the amplitude of the nerve action potential and age. Thus the normal ageing of a peripheral nerve can be demonstrated by electroneurography Electroneurographical signs of neuropathy of the median nerve were present in 33 % of the patients with pernicious anemia. Only 7 % of them had subjective symptoms. There was correlation between an abnormal electroneurogram and the occurrence of neurological symptoms. Neuropathy seems to be the rule rather than the exception in pernicious anemia, and an early sign rather than a late complication. N definite improvement was found after vitamin B_{12} therapy

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Effect of Heparin on Platelet Adhesiveness

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Owing to its prompt anticoagulant action heparin is firmly established as drug of choice for the treatment of acute thromboembolic disease. As a prophylactic agent its utility is limited by the need for frequent parenteral administration, a narrow therapeutic margin and a certain risk of serious untoward effects on continued use [23]. Despite these shortcomings heparin prophylaxis must be considered after surgery and in cases of recurrent or impending thrombophlebitis and thromboembolism. In such patients the tendency of blood platelets to adhere to one another and to foreign surfaces is often increased [2, 7, 15, 26, 33, 34, 39, 47, 51]. The treatment, therefore, should not only prevent intravascular clotting but should also restore normal platelet behavior. While the ability of heparin to achieve the former is generally acknowledged, its effect on platelet stickiness is still unresolved and a subject of conflicting reports.

In this paper *in vitro* effects of heparin on platelet adhesiveness are described. Since heparin may cause clumping and disintegration of platelets when added to whole blood or undiluted platelet-rich plasma (PRP) [9, 18, 25, 41] all experiments were conducted with diluted PRP in which platelet aggregation was considerably reduced. Adhesiveness of platelets was estimated by their ability to attach to the surface of human red cells which had been washed and treated with tannic acid [53].

Methods

Citrated PRP was obtained from human volunteers by conventional technique. It was diluted with imidazole buffer-saline pH 7.2 (IBS) to give cell counts of 6–8 10 /mm. Red

cells of the same donor were washed three times with 0.85% saline suspended in IBS at 1 ml of packed cells per 25 ml and incubated with an equal volume of tannic acid dissolved 1:20,000 in IBS for 15 min at 37°C. The cells were then washed once more, resuspended in IBS to match the count of the diluted PRP. Platelet suspension (0.2 ml) and saline-diluted heparin (0.1 ml) were mixed in siliconized glass tubes. Immediately thereafter 0.1 ml of the red cell suspension was added. After gentle mixing the tubes were incubated for 2 and 30 min at 37°C. A drop of the mixture was then spread on a polished glass slide. Smears were air-dried, fixed and stained with Leishman stain. They were evaluated under the microscope by counting the total number of platelets adhering to 500 red cells.

Heparin was used at various concentrations either alone or in combination with adenosine diphosphate (ADP), adenosine monophosphate (AMP), protamine sulfate, ethylenediamine tetraacetate disodium dihydrate (EDTA) and N-ethylmaleimide (NEM). All concentrations were calculated for the final mixture. In each experiment at least 5 control tubes were included in which heparin, the additional compound or both were substituted by corresponding volumes of saline. Variations of the mixing sequence of heparin, additional compound, platelets and red cells are described in the result section. Heparin-retarded plasma clotting time was determined by the method of POLLER [43] using Na citrate instead of K oxalate as anticoagulant.

Six New Zealand albino rabbits of both sexes weighing from 3.2 to 4.25 kg were injected intravenously with 1,000 units (U)/kg of heparin. Before and repeatedly after the injection blood samples were obtained from a marginal ear vein. They were collected into siliconized glass tubes containing Na citrate or into ordinary glass clotting tubes, kept on melting ice and spun within 1 h of collection at 3,000 rpm for 10 min at 4°C. Samples of rabbit plasma or serum (0.1 ml) were added to a mixture of platelets (0.2 ml) and red cell suspension (0.1 ml), mixed gently and incubated at 37°C for 2 min. Smears were prepared and processed as described. In two rabbits whole blood clotting time was determined before and repeatedly after heparin injection by a capillary tube method [28].

Materials

Heparin Likblum: Leo Pharmaceutical Products. ADP, NEM: Sigma Chemical Company. EDTA: L. Light & Co. AMP: Roche Products Ltd. Welwyn Garden City, courtesy Dr A. MORRISON. Protamin sulfate and Na citrate: The British Drug Houses Ltd. – Na citrate was used as 20% solution and 0.2 ml were added to 10 ml of blood.

Results

Effect of heparin. Platelet adhesiveness was markedly increased if heparin was present in the mixture tube at 1 U/ml or more (table I). This increase was not reversible after 30 min of incubation. At 0.2 U/ml or less heparin had no significant effect. With concentrations between 0.2 to 1 U/ml results were variable: adhesiveness was increased in some experiments and no effect was seen in others. Low adhesive counts were occasionally recorded with heparin concentrations from 0.02 to 0.1 U/ml. This finding was inconsistent and statistically not

Table I. Effect of heparin on platelet adhesiveness and plasma clotting time

Heparin U/ml	Number of platelets adhering to 500 red cells	Plasma clotting time min
25	264 ± 63	40+
2.5	231 ± 88	7
1	198 ± 47	5
0.5	178 ± 75	4
0.25	112 ± 26	3½
0.125	65 ± 27	3½
0.0625	94 ± 26	3½
0.03125	117 ± 44	3½
0	100 ± 29	2¾–3½

Final concentration

Mean ± standard deviation of 6 samples. Adhesive platelet counts with 1 U/ml of heparin and more are significantly higher than the saline controls (t test of Student, $p < 0.01$)

significant. A definite prolongation of plasma clotting time was present at 1 U/ml of heparin and more. 0.5 U/ml had a marginal effect and lower concentrations were inactive (table I) In several experiments the tanned red cells were incubated with heparin at final concentrations of 10 and 50 U/ml. The red cells were then washed resuspended in IBS and added to the platelet suspension. No increase in platelet adhesiveness was seen in comparison with samples containing red cells not previously treated with heparin.

Heparin and ADP If ADP was added to platelet-red cell mixtures to give final concentrations of 2×10^{-6} M or more an increase in platelet adhesiveness could be demonstrated [53] Heparin did not reduce this effect. On the contrary if marginally active concentrations of ADP and heparin were used their combined effect exceeded that of either component alone (table II)

Heparin and AMP AMP was tested at a final concentration of 5×10^{-3} M. It was added to the platelets and red cells either before or after heparin. Results of a typical experiment are summarized in table III It shows that the heparin induced increase in platelet adhesiveness was inhibited by AMP The effect was somewhat more pronounced if AMP was added to the mixture before heparin

Heparin and protamine sulfate Equal volumes of heparin and protamine sulfate solutions were mixed and 0.1 ml of the mixtures were added to 0.2 ml of platelets and 0.1 ml of red cell suspensions. Protamine sulfate

Table II. Combined effect of heparin and ADP on platelet adhesiveness

Experiment No.	Heparin U/ml	ADP molarity	Number of platelets adhering to 500 red cells	p
1	0.25	0	105 ± 27	–
	0.25	2 10^{-6}	202 ± 39	< 0.01
	0.5	0	79 ± 23	–
	0.5	2 10^{-6}	181 ± 50	< 0.01
	0	2 10^{-6}	91 ± 16	–
	0	0	67 ± 39	–
2	1	0	163 ± 24	–
	1	2 10^{-6}	237 ± 55	< 0.05
	1	1.5 × 10^{-6}	312 ± 64	< 0.01
	2	0	157 ± 27	–
	2	2 10^{-6}	296 ± 44	< 0.01
	2	1.5 10^{-6}	442 ± 92	< 0.01
	0	2 10^{-6}	167 ± 26	
	0	1.5 10^{-6}	205 ± 34	–
	0	0	134 ± 44	–

Final concentration

Mean ± standard deviation of 5-10 samples

t test of Student in comparison with group containing the same heparin concentration but no ADP. Controls contain equal volumes of saline.

Table III. Effect of AMP on heparin-induced increase in platelet adhesiveness

Heparin U/ml	AMP molarity	Mixing sequence of samples	Number of platelets adhering to 500 red cells	p
4	4 10^{-6}	AMP before heparin	66 ± 38	< 0.001
4	4 10^{-6}	heparin before AMP	107 ± 38	< 0.05
0	0		193 ± 50	
4	0		70 ± 23	< 0.001

Final concentration

Mean ± standard deviation of 5–10 samples

t test of Student in comparison with heparin controls.

abolished the effect of heparin on platelet adhesiveness if it was added at a ratio of 1 mg or more per 100 U of heparin (table IV). Protamin sulfate alone was tested at concentrations up to 1 mg/ml. No significant increase in platelet adhesiveness was noted. Some platelet aggregation occurred at the higher concentrations.

Table IV Effect of protamin sulfate on heparin-induced increase in platelet adhesiveness

Heparin U/ml	Protamin sulfate mg/ml[a]	Number of platelets adhering to 500 red cells	Protamin sulfate heparin ratio
2.5	0.1	95	4 mg 100 U
2.5	0.05	54	2 mg 100 U
2.5	0.025	39	1 mg 100 U
2.5	0.0125	171	0.5 mg 100 U
2.5	0.00625	220	0.25 mg 100 U
2.5	0	244	–

Final concentration

Mean of 3–4 samples. There was no overlapping of adhesive platelet counts between samples containing 0.025 mg/ml of protamin sulfate and more and the controls containing heparin only

Calculated for 100 U of heparin.

Table V Effect of platelet pre-incubation with NEM on heparin-induced increase in adhesiveness

Heparin U/ml	Pre-incubation of platelets	Number of platelets adhering to 500 red cells
10	NEM	45
10	saline	148
5	NEM	63
5	saline	245
2.5	NEM	35
2.5	saline	144
0	NEM	37 ± 11
0	saline	73 ± 36

Final concentration

Incubation 10 min at room temperature final concentration of NEM 5×10^{-4} M.

Controls contained equal volume of saline.

Mean of 3 samples each for 10 and 5 U/ml of heparin, 4 samples for 2.5 U/ml of heparin, 5 samples (± standard deviation) for controls containing no heparin. There was no overlapping of adhesive platelet counts between samples containing NEM-incubated platelets and corresponding samples containing heparin and saline-incubated platelets.

Heparin and EDTA EDTA at final concentration of 5×10^{-3} M was combined with heparin dilutions ranging from 1.25–25 U/ml. 0.1 ml of the mixtures were added to 0.2 ml of platelets and 0.1 ml of red cell suspension. No significant inhibition of the heparin effect was noted. EDTA alone markedly reduced platelet adhesiveness [53]

Heparin and NEM For the evaluation of NEM a substance which blocks SH-groups, platelet suspensions were first incubated with the compound for 10 min at room temperature. The NEM concentration was 5×10^{-4} M. Platelets treated in this manner failed to show an increase in adhesiveness if heparin was added at final concentrations of 2.25 to 10 U/ml (table V) If, however the tanned red cells were incubated with the SH blocker washed and then added to untreated platelet suspension and heparin no inhibition of the heparin effect was demonstrated

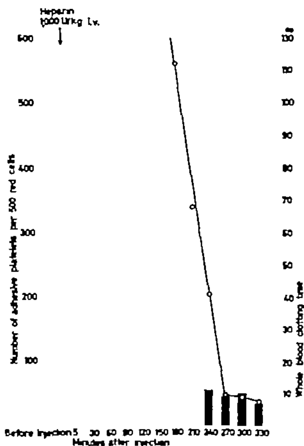


Fig 1 Whole blood clotting time (open circles) and effect of serum samples on *in vitro* platelet adhesiveness (black bars) in rabbit injected with 1,000 U/kg of heparin i.

Plasma and serum of heparin-treated rabbits. A marked increase in platelet adhesiveness was produced with all plasma and serum samples obtained immediately and up to 1 h after the heparin injection. In some animals the effect lasted for up to 210 min. Figure 1 shows the findings in one rabbit in which whole blood clotting time and the effect of serum samples on *in vitro* platelet adhesiveness were determined. The whole blood clotting time was markedly prolonged and returned to near normal 270 min after the heparin injection. The effect on platelet adhesiveness could be demonstrated for up to 210 min.

Discussion

At physiologic pH platelets have a high negative net charge [1, 13]. The electrostatic surface charges determine, to a considerable extent, the relationship of platelets to each other and to foreign surfaces [49]. It is to be expected, therefore, that electrically charged macromolecules could affect platelet aggregation and adhesiveness. In the case of heparin this possibility has been studied extensively. Since heparin is a highly negatively charged molecule it is conceivable that it might reduce platelet adhesiveness and aggregation, perhaps by increasing the negative charges of the platelets or by binding Ca ions which are essential for platelet aggregation. Heparin might, furthermore, antagonize thrombin formation on the platelet surface which is thought to be a factor in platelet aggregation [40]. There are, indeed, several papers in the literature which report that heparin inhibited platelet aggregation and adhesiveness *in vitro* [14, 32, 38] and thrombus formation in mechanically damaged blood vessels of rats [5] or extracorporal shunts in dogs, cats and monkeys [3]. However, most studies concerned with the effect of heparin on platelets failed to demonstrate a reduction in aggregation and adhesiveness. On the contrary, an increase in adhesiveness and the tendency to clump was often observed. Heparin added to blood or PRP was found to cause platelet clumping and destruction [9, 11, 18, 19, 25, 30, 41]. Heparinized plasma caused aggregation of added platelets [10] and heparin increased platelet adhesiveness to glass [10, 21, 37]. Intravenous injection of heparin into dogs caused a sharp drop in platelet count and appearance of platelet clumps [12, 18, 44]. Similarly, i.v. injection into hamsters produced platelet emboli which could be visualized in blood vessels of

the cheek pouch [20 31] and in blood obtained directly from the heart [8] Platelet clumps were also observed in rabbits in which heparin treatment was combined with small i.v doses of Na laurate [52] Heparin did not reduce white body formation following mechanical irritation of blood vessels of rabbits and guinea pigs [27] and platelet plugs, although fragile ones, continued to be formed in injured mesenteric veins of rats treated with very high doses of heparin [54]

The present *in vitro* study also demonstrated an increase in platelet adhesiveness by heparin at concentrations exceeding 0.5 U/ml. It is interesting to compare this effect with that of ADP which plays a key role in the mechanism of platelet aggregation and produced a marked increase in adhesiveness in the platelet red cell system [53] AMP inhibited this effect of ADP probably by competing for specific binding sites on the platelet surface [4 46] The heparin effect on platelet adhesiveness was also inhibited by AMP suggesting that heparin may affect the same binding sites. This possibility gains further support from the observation that combination of marginally active concentrations of ADP and heparin produced a marked increase in adhesiveness. A synergistic effect of ADP and low heparin concentrations was also demonstrated in experiments measuring platelet adhesiveness to glass [50]

A further similarity between ADP and heparin could be demonstrated with NEM, a specific inhibitor of SH-groups. Platelets contain reactive SH-groups [17] which are important for platelet adhesiveness and aggregation [24 45] Blockage of these groups inhibited platelet adhesiveness [53] and neither ADP nor heparin could overcome this effect. The treatment of the tanned red cells with NEM did not affect platelet adhesiveness. Thus, the reactive SH-groups of the red cells which are abundant [17] are not essential for the adhesion of platelets to the red cell membrane.

The effect of heparin on platelet adhesiveness differed from that of ADP in that it was not readily reversible, ADP is rapidly metabolized [29] and soon loses its effect. Heparin is not so quickly destroyed and can affect platelets over a longer period of time. The experiments with EDTA uncovered another difference ADP induced increase in platelet adhesiveness was strongly inhibited by the chelator [53] whereas the heparin effect was not significantly altered. Divalent cations are essential for secondary bridging of aggregated platelets, a process which also requires fibrinogen [6, 35 46] The ADP-induced platelet

adhesion needs this reinforcement whereas the more stable heparin binding can apparently proceed without it.

Heparin is not the only charged macromolecule which affects platelets. Na polyanetholsulfonate (Liquoid® Roche) caused both platelet clumping and increased adhesiveness [53] and produced thrombosis after injection into animals [16] Moreover various dextran sulfates were shown to cause platelet clumping [42] Protamin sulfate, however a positively charged macromolecule did not increase *in vitro* platelet adhesiveness, although some platelet aggregation was noted. Thus, platelet charges and behavior were changed by this agent, as it was also reported by SCHNEIDER *et al.* [48] In addition protamin sulfate inactivated the heparin effect on platelet adhesiveness. The protamine sulfate heparin ratio required to abolish the heparin-induced increase in adhesiveness was the same as that necessary to inhibit the anticoagulant action of heparin.

Is the described *in vitro* effect of heparin on platelet adhesiveness of clinical significance? From the experimental evidence reported in this paper it is clear that increased platelet adhesiveness does not only occur after excessive heparin doses but also with concentrations within conventional therapeutic range. This aspect of the heparin spectrum is often overlooked because the laboratory tests designed to measure clot formation are dominated by the potent anticoagulant action of the drug But when changes in platelet adhesiveness were especially looked for other aspects of the heparin action could sometimes be uncovered. For example, platelet adhesiveness to glass 45 min after i.v injection of 200 mg (about 26,000 U) of heparin was often increased [32] Repeated subcutaneous injections of moderate to large doses of heparin also produced some increase in platelet adhesiveness [36] A drop in platelet count, perhaps related to platelet clumping, was seen after i.v and s.c. heparin injections in man [18] This effect was not confirmed by others [44] Considering all the evidence it appears probable that platelet aggregation and adhesion to endothelium are frequent sequelae of heparin injection. Because of reduced coagulability of the blood these events are probably of little clinical significance They may however present a potential hazard if therapy with heparin is not continuously maintained at an effective level. Thus oscillation between periods of high heparin blood levels with formation of small platelet aggregates and periods of low levels with insufficient inhibition of coagulation may create favorable conditions for the development of thromboembolic

complications. The study also underlines the urgent need for a reliable drug which reduces platelet adhesiveness and could be administered alone or in combination with heparin and other anticoagulants.

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Summary

Heparin increased platelet adhesiveness to tannic acid-treated human red cells *in vitro* at concentrations of 1 U/ml and more. This effect was inhibited by AMP, enhanced by ADP but not affected by EDTA. Protamin sulfate inactivated heparin at a ratio of 1 mg per 100 U of heparin. Heparin did not increase adhesiveness of platelets which had been treated with NEM, a blocker of reactive SH-groups. Plasma and serum of rabbits injected with 1000 U/kg of heparin i.v. increased platelet adhesiveness when added to the platelet-red cell system. This effect lasted for up to 210 min and paralleled roughly the *in vivo* prolongation of whole blood clotting time.

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Zur Darstellung der Transformationsfähigkeit von Lymphozytenkulturen bei verschiedenen Erkrankungen mittels Feulgen Zytophotometrie[1]

E. Ambs, J Chiesebro und B. Lagerlöf

Nowell konnte im Jahre 1960 beobachten, dass das Phytohämagglutinin (PHA) ein Protein aus einer Bohnenart *(Phaseolus vulgaris)* Leukozytenkulturen zu einer Zell Transformierung stimuliert. Inzwischen sind viele Agentien bekannt geworden, die eine Transformation von kleinen Lymphozyten in unterschiedlichem Ausmass bewirken. Es entstehen die sogenannten blastenartigen Zellen, die sich auf eine Mitose vorbereiten und diese auch erreichen. Dieser Effekt hat bei Zytologen und Immunologen zunehmend an Interesse gewonnen, worüber an anderer Stelle aus dem Blickwinkel der Klinik zusammenfassend berichtet werden soll [5]

Man kann eine spezifische und unspezifische Stimulierung unterscheiden. Die unspezifische Stimulierung ist praktisch bei allen menschlichen Individuen möglich. Das klassische Beispiel ist das PHA, das von allen Agentien die höchste Transformationsrate erreicht. Die spezifische Stimulierung setzt dagegen die vorherige Auseinandersetzung des untersuchten Lymphozytenspenders mit dem entsprechenden Antigen voraus. Viele Autoren denken bei dieser *in-vitro*-Stimulierung an Parallelen zu secondary response und Allergie vom verzögerten Typ. Während bei der unspezifischen Stimulierung unter der Transformation in den Zellen eine γ-Globulin-Synthese nachweisbar wird, entstehen nach spezifischer Stimulierung die entsprechenden Antikörper Sowohl die Transformation, als auch diese Synthesen sind *in vitro* durch Zusatz von Corticosteroiden hemmbar. In Lymphozytenkulturen von Patienten mit einer Allergie gegen Penicillin, Hydantoine oder Pollen werden durch die entsprechenden Agentien Transformation und Antikörperbildung ausgelöst. Schliesslich gibt es Erkrankungen, bei denen zelluläre Transformation und Proteinsynthesen

[1] Herrn Dr Bo Johansson, Radiumhemmet, Karolinska Sjukhuset, Stockholm, sind wir für seine liebenswürdigen Bemühungen zu grossem Dank verbunden, an seinem Krankengut die vorliegenden Studien durchführen zu können. Die Arbeit wurde unterstützt von Fonds des Karolinska Institutets (Gunnel och Svante Högboms Stiftelse, Stiftelsen Therese och Johan Anderssons Minne)

herabgesetzt sind. Hierhin gehören die Lymphozyten bei angeborenem Mangel des Thymus und bei angeborenen A- und Hypo-γ-Globulinämien. Falls die Transformation auf unspezifische Stimulierung erhalten ist, so fehlt hier jedoch die γ-Globulin-Synthese. Bei spezifischer Stimulierung bleibt sowohl die Transformierung als auch die Antikörperbildung aus.

Eine herabgesetzte Transformationsfähigkeit besteht auch bei einer Gruppe von Erkrankungen, die DAMASHEK [11] als immunoproliferative Erkrankungen zusammengefasst hat, wobei er den Lymphozyten eine zentrale Stellung innerhalb der Immunreaktionen einräumt. Davon interessieren hier zwei Untergruppen: die lymphoproliferativen Erkrankungen, unter die die chronische lymphatische Leukämie (CLL) sowie die Lymphosarkomatosen fallen, und die retikuloproliferativen Erkrankungen mit Morbus Hodgkin und Sarkoidose (M. Boeck).

Allen Erkrankungen gemeinsam ist die Immuninkompetenz und Infektionsneigung sowie die verzögerte Abstoßung von homologen Hauttransplantaten [33]. Während bei der CCL die Hypo-γ-Globulinämie [6] und die mangelhafte Bildung von Antikörpern [28, 34] im Vordergrund steht, tendiert der Morbus Hodgkin zur Störung der Allergie vom verzögerten Typ [4]. Die Erscheinungen überlappen sich jedoch bei den einzelnen Krankheitsbildern [37?–40], wobei der Verlauf und das Stadium zu berücksichtigen sind. Bei diesen Erkrankungsgruppen ist ferner eine herabgesetzte Transformationsfähigkeit der Lymphozyten bekannt.

Die vorliegende Arbeit hat die Aufgabe, das Transformationsvermögen quantitativ darzustellen. Eine Erfassung der Transformationsrate ist am einfachsten möglich durch das Auszählen eines Differentialzellbildes der verschiedenen Transformationsstufen in der Kultur [14], wobei allerdings fließende morphologische Übergänge zu berücksichtigen sind [30]. Zuverlässiger ist eine autoradiographische Untersuchung, wobei sich der Einbau radioaktiv markierten Thymidins zur Darstellung der DNS-Synthese empfiehlt. So haben beispielsweise YOFFEY *et al.* [47] die morphologische Differenzierung mit der Autoradiographie und mikrospektrographischen DNS-Messungen kombiniert. In unseren Untersuchungen wurde durch Feulgen-Photometrie und Karyometrie an einzelnen Zellen die Häufigkeitsverteilung der DNS-Werte und der mittleren Kerndiameter aufgestellt und damit die Transformationskinetik in Lymphozytenkulturen quantitativ demonstriert.

Material

Als *Kontrollen* wurden die Lymphozyten aus dem Blut dreier Blutspender mit normalen hämatologischen Werten untersucht (Serie 6, 7, 8).

Patienten. Die Serien 9, 10, 15 umfassen das Blut von 3 Patienten mit CLL. *9* 59jähriger Patient, überwiegend tumorös-medulläre Form, Erkrankung vor 4 Jahren und 2 Monaten diagnostiziert. Vergrößerung der Hals- und Mediastinal-Lymphknoten. Vor 4 Jahren Chemotherapie, Corticosteroide und Röntgenbestrahlung. 13,2 g/100 ml Hb, 10200 Leukozyten/mm³ mit 85% Lymphozyten und 15% Segmentk. *10* 56jähriger Patient, Erkrankung vor 6½ Jahren diagnostiziert. Seit 3 Monaten Sendoxan und Corticosteroide. Jetzt ausgedehnte Mageninfiltrationen. 10,8 g/100 ml Hb, 14000 Leukozyten/mm³ mit 81,5% Lymphoz., 1% Basoph., 2% Stabk., 13,5% Segmentk., 2% Monoz.; 115000 Thrombozyten/mm³. *15* 75jähriger Patient, Erkrankung vor 10 Jahren diagnostiziert, vor 3 Wochen Röntgenbestrahlung der Milz (500 r). 13,0 g/100 ml Hb, 16400 Leukozyten/mm³, 80,5% Lymphoz. und 19,5% Segmentk.

Bei den Patienten mit Morbus Hodgkin handelt es sich um eine Patientin (11) von 36 Jahren, deren Erkrankung seit 12 Jahren bekannt ist. Sie wurde in früheren Jahren röntgenbestrahlt und befindet sich seit 1 Jahr in einer Remission ohne Beschwerden und klinische Zeichen. 13,2 g/100 ml Hb, 4000 Leukozyten mit 5% Eos., 1% Stabk., 40% Segmentk., 33% Lymphoz. Die andere Patientin (13) ist 43 Jahre alt, die Erkrankung seit 20 Jahren bekannt. In dieser Zeit wurden alle therapeutischen Möglichkeiten ausgeschöpft. Es handelt sich jetzt um ein generalisiertes Stadium. Die letzte Therapie mit Natulan und Corticosteroiden liegt etwa 3 Monate zurück. 4000 Leukozyten/mm³ mit 2% Eos., 73% Segmentk., 19% Lymphoz.

Untersuchung 12 stammt von einer 67jährigen Patientin, bei der seit 6 Jahren ein Lymphosarkom (lymphozyto-lymphoblastoid) der Submandibularregion bekannt ist. Behandlung mit Röntgenstrahlen, Zytostatika und Corticosteroiden, letzte Behandlung vor 4–5 Monaten. Blutbild unauffällig. Der Patient 14 mit Retikulumzellsarkom der linken Lendenregion ist 42 Jahre alt; seine Erkrankung ist seit 4½ Jahren bekannt. Letzte Röntgenbestrahlung vor 1½ Jahren. 12,8 g/100 ml Hb, 3800 Leukozyten/mm³ mit altersgemäßem Differentialblutbild.

Methodik

Die Kulturen enthielten bei Beginn der Kontrollen (6, 7, 8) 1,1, 1,0 bzw. $0,8 \times 10^6$/ml Zellen, bei den CLL (9, 10, 15) 1,3, 1,2 bzw. $2,2 \times 10^6$/ml Zellen, bei den Kulturen von Morbus Hodgkin (11, 13) 0,5 bzw. $1,6 \times 10^6$/ml Zellen, beim Lymphosarkom (12) 2,0 und beim Retikulumzell-Sarkom (14) $1,6 \times 10^6$/ml Zellen. Von jeder dieser Kulturen wurde ein Doppelversuch angesetzt.

Kulturtechnik. Zehn ml Venenblut wurden beim Abnehmen mit 0,15 ml Heparinlösung vermischt und in ein Reagenzglaschen gegeben. Hier wurden 1,5 ml einer 6%-Dextranlösung in physiologischer Kochsalzlösung zugefügt. Nach Durchmischung wurde das Glas eine Stunde lang bei 37°C in einem Winkel von 45°C gelagert, so dass sich die Zellen absetzen konnten. Sodann wurde mit einer Pasteur-Pipette das überstehende, leukozytenhaltige Plasma abgehebert und bei 1500 U/min 10 min lang zentrifugiert. Der Überstand wurde verworfen, die Zellen in 1 ml Kulturmedium aufgenommen (Parker TC 199 und 15% Kalbserum). Danach wurde die Zellzahl in der Suspension ermittelt und solange weiter verdünnt, bis eine Zellkonzentration von ungefähr $1,2 \times 10^6$/ml erreicht war. Auf 5 ml Kulturflüssigkeit wurden abschließend 0,1 ml Difco Bacto Phythämagglutinin-P zugegeben. Die Kulturen wurden in sterilen Glasröhrchen bei 37°C inkubiert.

Präparationen. Zu Beginn und nach 24, 48 bzw. 72 h wurden mittels einer sterilen Injektionsspritze aus den Kulturen kleine Proben von 0,1–0,2 ml entnommen und daraus die Zellen mittels einer speziellen Zentrifuge direkt auf Objektträger zentrifugiert (Shandon Scientific Co. Ltd., London), und zwar 10 min lang bei 1500 U/min [45]. Dabei wurden immer doppelte Präparate angefertigt, wobei die einen nach May-Grünwald-Giemsa, die

anderen nach FEULGEN [16] in einer speziellen Modifikation [2] gefärbt wurden. Die Versuchsanordnung war so eingerichtet, dass alle Bedingungen möglichst gut reproduzierbar wurden.

Messung der DNS-Menge und Karyometrie an der Einzelzelle. Die Feulgen-Photometrie [2] wurde nach einer von ADAMS [1] entwickelten Methodik durchgeführt. Die gefärbten Zellen wurden bei 560 nm mikrophotographiert. Die Abbildung der Zelle auf dem Film wurde nun innerhalb einer geeigneten Blende gegen den benachbarten Schwärzungshintergrund photometriert, wobei eine angeschlossene Recheneinrichtung den Extinktionswert der einzelnen Zelle automatisch registrierte, woraus dann die DNS-Menge der Einzelzelle in relativen Einheiten errechnet wurde. Zur Eichung diente das Ausmessen von mindestens 50 frisch ebenso präparierten und gefärbten Thymozyten der Maus. Hier befindet sich die durchschnittliche DNS-Menge der Einzelzelle im gleichen Grössenbereich, die Verteilung der Werte ist jedoch sehr eng, so dass der diploide Gipfel sehr gut zu erkennen ist [1, 2]. Die photographische Abbildung diente gleichzeitig zur Messung des mittleren Kerndiameters. Zur quantitativen Erfassung der Transformationsprozesse wurde von beiden Werten von je 100 mononukleären Zellen die Häufigkeitsverteilung bei 0, 24, 48 und 72 h aufgestellt.

Ergebnisse

Alle drei Kulturen von Blutspendern [6, 7, 8] zeigen die bekannten Veränderungen der PHA Kulturen. Nach 72 h sind in allen Kulturen Mitosen aufgetreten. Die individuelle Entwicklung ist in Abbildung 1 als Beispiel einer solchen Kultur zu erkennen, der diploide Gipfel nimmt mehr und mehr ab. Dafür werden die Zellen mit hyperdiploiden und tetraploiden Werten häufiger. Unter der Transformation verschiebt sich ausserdem die Verteilungskurve der Kerndiameter zu grösseren Werten. Dabei ist zunächst nach 24 h das bekannte Schrumpfungsphänomen von Zellen in flüssigen Kulturen zu sehen. Schliesslich macht sich bei dieser Zentrifugationstechnik sehr stark die agglutinierende Wirkung des PHA bei den transformierenden Zellen bemerkbar [20, 43]. In Tabelle I sind die Durchschnittswerte von je 100 Zellen zu verschiedenen Zeiten an DNS erreicht, wodurch das Ausmass der DNS-Synthese noch einmal deutlich wird. Tabelle II zeigt die gleichen Angaben für den mittleren Kerndiameter. Hier finden sich auch entsprechende Angaben für die übrigen Zellpopulationen.

Die 3 Populationen von CLL [9, 10, 15] verändern die Häufigkeitsverteilung der DNS-Werte und der Kerndiameter praktisch nicht (Abb. 2). Kultur 9 zeigte anfangs insgesamt grosse und unreif wirkende Lymphozyten, die nach 72 h etwa die gleichen Grössenverhältnisse, keine Nucleoli und durchwegs ein sehr helles Zytoplasma boten. Nur vereinzelt sah man mittelgrosse, typische Blasten. Bei Kultur 10

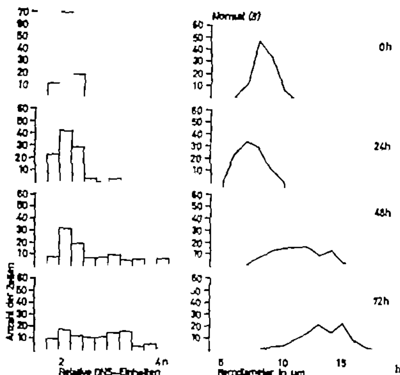


Abb. 1 Häufigkeitsverteilung von (transformierten) Lymphozyten normaler Kontrollpersonen nach gleichen Klassen bezüglich der DNS-Menge ( ) und des mittleren Kerndiameters (b) (Untersuchungsreihe 8)

handelte es sich anfangs um sehr kleine und dichte Lymphozyten, die nach 72 h eher die Tendenz zur Vergrößerung zeigten, wobei aber das Zytoplasma wiederum auffallend hell blieb Auch hier waren keine Mitosen und Nucleoli aufgetreten nur einige wenige blasten artige mittelgrosse Zellen Bei Kultur 15 lagen ebenfalls sehr kleine Lymphozyten vor die nach 72 h nahezu unverändert schienen.

Zwischen diesen beiden Gruppen der Lymphozyten von Blutspendern und von Patienten von CLL mit praktisch fehlender Transformationsfähigkeit lagen die anderen Untersuchungen mit herabgesetzter Transformationsrate. *Die erste Kultur von Morbus Hodgkin* (11) zeigte eine befriedigende Transformation (Abb 3) liess aber keine Mitosen erkennen. Die zweite Kultur (13) fiel dadurch auf, dass im Verlauf relativ viele Neutrophile vorhanden waren, die Zahl der Lymphozyten aber schnell abnahm. Soweit noch vorhanden, zeigten sie überwiegend einen zusammengesinterten Kern Nur wenige waren

Tabelle I. Durchschnittliche DNS-Menge der Kerne in relativen Einheiten (= 100)

	0 h	24 h	48 h	72 h
Normal (6)	129,60	130,90	147,93	162,03
Normal (7)	127,33	128,49	138,00	159,63
Normal (8)	129,27	131,90	159,03	167,30
CLL (9)	127,55	129,93	130,36	127,59
CLL (10)	125,01		126,10	129,82
CLL (15)	129,10	131,06	129,46	152,86
M. Hodgkin (11) (Remission)	127,68	126,48	136,78	153,24
M. Hodgkin (13) (generalisiert)	143,30		–	135,53
	(= 20)			(= 33)
Lymphosarkom (12)	126,36		132,02	144,54
Retikulumzellsarkom (14)	130,14		147,87	150,43

Tabelle II. Durchschnittlicher mittlerer Kerndiameter in µm (= 100)

	0 h	24 h	48 h	72 h
Normal (6)	8,66	6,56	10,09	11,7
Normal (7)	8,53	7,18	10,87	11,64
Normal (8)	8,41	7,24	11,44	13,47
CLL (9)	10,27	9,53	10,23	9,51
CLL (10)	8,51	8,99	8,49	9,29
CLL (15)	9,23	9,21	9,12	9,12
M. Hodgkin (11) (Remission)	9,39	9,48	10,59	10,58
M. Hodgkin (13) (generalisiert)	8,94			9,42
	(= 20)			(= 33)
Lymphosarkom (12)	10,66		9,64	10,02
Retikulumzellsarkom (14)	8,34		11,14	10,97

erhalten, von denen wiederum einige eine beginnende Transformierung, aber nur bis zu mittelgrossen Formen, erreichten. In dieser Kultur liessen sich daher nur sehr wenige Zellen ausmessen.

Eine herabgesetzte Transformationsrate wiesen auch die Kulturen der Patienten mit Lymphosarkom (12) (Abb 4) und Retikulumzellsarkom (14) (Abb 5) auf, von denen nur letztere mehrere Mitosen erkennen liess.

Alle Untersuchungsergebnisse sind in Abbildung 6 zusammengefasst, auf der die DNS-Werte der untersuchten Zellen zu den verschiedenen Zeitabschnitten gemittelt sind. Hier ist die Transformationskinetik gut zu erkennen. Die Lymphozytenkultur des Morbus Hodgkin (13), bei dem die Zellen überwiegend zugrundegegangen sind, ist gestrichelt eingezeichnet. Das Verhalten der «Lymphozyten» bei den

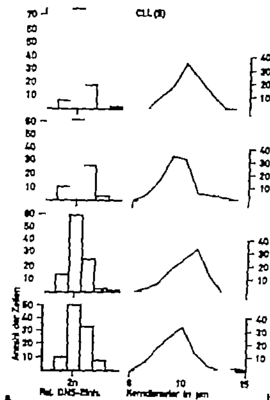


Abb. 2. Häufigkeitsverteilung von Lymphozyten einer chronischen lymphatischen Leukämie nach Klassen bezüglich der DNS-Menge (a) und des mittleren Kerndiameters (b) (Untersuchungsreihe 9)

hier untersuchten Fällen von CLL unterscheidet sich von der Transformationsfähigkeit der Lymphozyten von normalen Kontrollpersonen und von Patienten mit anderen Erkrankungen dieser Gruppe.

Diskussion

Während die Lymphozytenkulturen der 3 Kontrollpersonen übereinstimmend eine sehr gute Transformationsfähigkeit zeigten, trat diese in den 3 Kulturen von Patienten mit chronisch lymphatischer Leukämie nur bei sehr wenigen Zellen ein. Mitosen wurden dabei nicht beobachtet. Die Transformationsrate bei den Patienten mit Morbus Hodgkin, Lymphosarkom und Retikulumzell-Sarkom war herabge

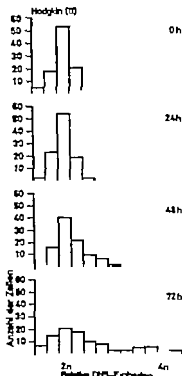


Abb. 5. Häufigkeitsverteilung von (transformierten) Lymphozyten nach gleichen Klassen bezüglich der DNS-Menge bei Morbus Hodgkin (Patient 11)

setzt. Eine Kultur eines Patienten mit Lymphogranulomatose im generalisierten Stadium zeigte ein Absterben der Zellen wovon nur wenige Zellen ausgenommen wurden, die ganz vereinzelt eine beginnende Transformierung besaßen. Das entspricht den Erfahrungen in der Literatur

Die Verteilung der DNS-Werte von Lymphozyten bei der *chronischen lymphatischen Leukämie* weist ausschließlich diploide Werte auf. GAHRTON und FOLEY [19] sahen bei ihren Messungen, daß die DNS-Werte durchschnittlich 10% höher lagen als bei normalen Lymphozyten was hier nicht bestätigt werden konnte. Sie beobachteten ferner einen niedrigen Gehalt an Nucleotiden und RNS sowie niedrige Werte der Zellmasse. In der PHA Kultur änderte sich in unseren Versuchen die Verteilung der DNS-Werte und der mittleren Kerndiameter kaum. Über gleiche Ergebnisse einer stark herabgesetzten

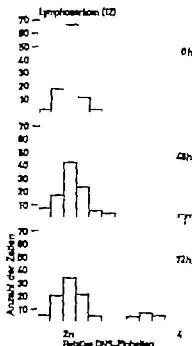


Abb. 4 Häufigkeitsverteilung von (transformierten) Lymphocyten nach gleichen Klassen bezüglich der DNS-Menge bei Lymphosarkom (Patient 12)

Transformation wird von den meisten Untersuchern berichtet [12 13 22 26, 34 36 37 38, 39 44 46] sowohl nach unspezifischer als auch spezifischer Stimulierung wobei meist keine Mitosen auftraten.

Auf ein Antigen, mit dem sich ein Patient während seines Lebens schon früh auseinander gesetzt hat (*E. coli*) folgt jedoch eine sehr viel grössere Reaktion als beispielsweise auf Salmonellenantigene, mit denen der Patient erst unter der Testung den ersten Kontakt bekommt [9] Die reduziert oder fast erloschene Transformationsfähigkeit ist mit einer Störung der Globulinsynthese [18] jedoch mit einem Anstieg der Aktivität der Laktatdehydrogenase verbunden, der nicht durch Aktinomycin-D gehemmt werden kann [37] Auch der zytotoxische Effekt dieser Lymphocyten in Kultur ist herabgesetzt [27] Viele Autoren nehmen keine Zusammenhänge zwischen der Höhe der Leukocytenzahl im Blut [35, 39 41], bzw zwischen dem Krankheitsbeginn [6] und der Transformationsfähigkeit an. Bernard [7] beobachtete die tiefste bei hohen Leukocytenwerten und vermutete zunächst, dass neben den pathologischen Zellen noch normale Lymphocyten vorliegen könnten, auf die die Umwandlungsfähigkeit beschränkt sei. Indessen sind bei der CLL auch transformierte Zellen mit abnormalen Chromosomenverhältnissen gefunden worden [17]. Es besteht eine signifikante Korrelation zwischen dem Logarithmus der Anzahl der Zellen im peripheren Blut und dem Prozentsatz an Lymphocyten, die in der Kultur Thymidin inkorporieren, und zwar sowohl bei den behandelten als auch bei unbehandelten Fällen

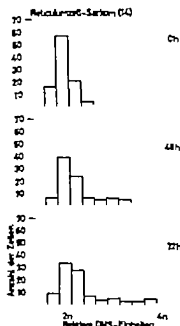


Abb. 5. Häufigkeitsverteilung von (transformierten) Lymphozyten nach gleichen Klassen bezüglich der DNS-Menge bei Reticulumzellsarkom (Patient 14)

[41]. Signifikante Unterschiede bei unbehandelten und behandelten Patienten scheinen nicht zu bestehen. Erst bei massiver Röntgenbestrahlung und zytostatischer Behandlung, bei denen die Zellzahlen auf normale oder subnormale Werte abfallen, ist die Transformationsfähigkeit zusätzlich fassbar herabgesetzt [6, 41]

Bei der Gruppe der *Lymphosarkome* finden sich sowohl Angaben über eine herabgesetzte Transformation der Blutlymphozyten in der PHA Kultur [26, 36] als auch Beobachtungen über eine gleiche Transformierung wie in Normalkulturen [39] Wahrscheinlich ist das unterschiedliche Verhalten auch von der Verlaufsphase abhängig [44] Auch Zellen mit chromosomalen Anomalien können sich umwandeln [17]

Beim *Morbus Hodgkin* reichen die Beobachtungen von einer normalen über eine nur mäßig bis zu einer stark herabgesetzten Transformationsfähigkeit der Lymphozyten [3 10 12, 23 25 26 39 42] bis zum Absterben der Zellen in der Kultur [39 44] Überwiegend wird jedoch von einer herabgesetzten Reaktion auf PHA berichtet. Wenn den weniger aktiven Kulturen nach 24 h ein RNS-Extrakt aus Leuko-

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If done efficiently and rapidly filtration of the dye mixtures can be carried out in the light, thus eliminating the inconvenience of finding dark room to carry out the filtration. Towels can be placed around the Koplin jars t cut down light exposure while filtering. Immediately after filtering the jars should be placed in dark cupboard or drawer until ready for use. Stained slides stored for greater than 30 days showed diminution of staining of the more weakly reacting cells.

The staining reactions of peripheral blood and bone marrow smears from seven cases of acute lymphatic leukemia (ALL) and eight cases of acute myelogenous leukemia (AML) were studied. Smears from patients with chronic lymphatic leukemia (CLL) chronic myelogenous leukemia (CML) and multiple myeloma (MM) were also observed.

Peripheral blood smears, direct bone marrow smears and/or bone marrow concentrates were used in the investigation.

Results

Mature neutrophils showed no nuclear staining The cytoplasmic granules, however stained brown and appeared distributed throughout the cell as normally seen in WRIGHT's stained preparations as relatively fine stippling Because no counterstain can be used, the neutrophils were recognized by their stippled cytoplasm and lobulated nucleus.

Mature eosinophils also showed a negative nuclear reaction. Granular staining in the eosinophils was very intense and the cell could easily be recognized by the deep brown, large granules densely packing the cytoplasm around the bilobed nucleus.

Monocyte granules also stained positively with a negative nuclear reaction.

Mature basophils, because they are found in low percentages in normal blood, were not easily recognized. However in chronic myelogenous leukemia where the basophil percentage is increased, basophils were seen showing a granular staining reaction similar to that observed in the eosinophils. The granules were fewer in number than in eosinophils, but also showed an intense deep brown staining reaction. The nucleus of the basophils showed no ASA.

Nuclear ASA was evidenced only in the lymphocytes of normal blood. The staining reaction yielded a pale to bright pink colored nucleus. If nucleoli were present, they stained a deeper pink and were easily seen. The cytoplasm of the lymphocyte showed a negative to *faint pink reaction*

The platelets were easily noted as small positively staining bodies. The granules in the platelets stained a brown color Megakaryocytes as seen in the bone marrow smears had a light pink cytoplasm and dark granules. The nucleus of the megakaryocyte stained light to bright pink

indicating a positive reaction. Nucleoli were visible as positively staining bodies.

Macrophages and histiocytes also had a positive nucleus. Fat on marrow smears showed no ASA.

On investigating acute lymphatic processes, it was found that nearly all lymphoblasts and immature lymphocytes possessed nuclear ASA. The intensity of the staining reaction varied from cell to cell, but in all blasts, prominent nucleoli were noted. The staining varied from a faint gray to a deep red color in these cells. The monotonous blood picture which is noted normally in WRIGHT's stained smears of chronic lymphatic leukemia was not seen with the arylsulfatase staining method. Most of the lymphocytes showed nuclear ASA, but of varying intensity. Unlike the cells in acute lymphatic leukemias, lymphatic cells of chronic lymphatic leukemia did not stain as intensely a deep red color. The cells were pale pink to grayish-pink, but it is certain that the mature lymphocytes of chronic lymphatic leukemia possessed nuclear arylsulfatase.

Although previous investigators [2, 3] have found the nuclei of myeloblasts to be free of arylsulfatase, this investigation of eight cases of acute myelogenous leukemia, including both treated and untreated patients revealed arylsulfatase to be present in the nuclei of some myeloblasts (fig. 1). The staining of the myeloblasts was not uniform. Some contained a high degree of activity while others exhibited a faintly positive to completely negative reaction. The identification of cells as myeloblasts was confirmed by comparison of the arylsulfatase stained smears with WRIGHT's stained smears and noting percentages of blasts and in some cases the presence of Auer rods. Nucleoli were also visible and stained darker than the nucleus itself as in the lymphatic leukemias. In cases where Auer rods were seen in the WRIGHT's stained smears, no Auer rods were identified in the slides stained for arylsulfatase suggesting the possibility that Auer rods contain no ASA.

Two patients with CML were studied: one in a blastic crisis and one with a typical CML blood and bone marrow picture. Both patients were Philadelphia chromosome positive. In the patient with a preponderance of developing granulocytes of all types, the cells of the granulocytic series all possessed arylsulfatase negative nuclei. The granules of these cells stained as the granules of normal granulocytes. The patient in blastic crisis showed, as in the acute myeloid process, positive nuclei, most of them being weakly positive but containing

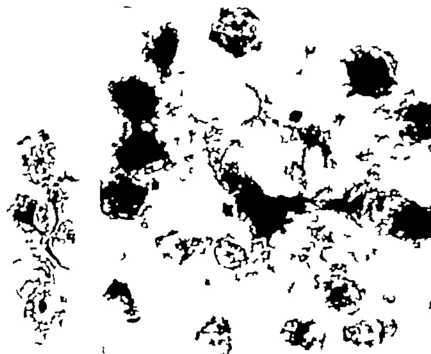


Fig 1 Bone marrow smear from patient with acute myelogenous leukemia demonstrating positive arylsulfatase staining in the nuclei and nucleoli of myeloblasts (× 1,000)

Fig 2 Bone marrow smear demonstrating positive arylsulfatase reaction in erythroid forms from case of erythroleukemia (× 1,000)

moderately positive nucleoli. The developing granulocytes present stained similarly to those in the first case

One case of previously diagnosed chronic myelogenous leukemia which at the time of the investigation show a marked preponderance of abnormal erythroid forms, predominantly in the earlier stages of development, was also studied. Slides stained for arylsulfatase revealed the nuclei of many of the erythroid precursors to be intensely arylsulfatase positive (fig 2) Almost all the cells had some nuclear activity most of them staining deep pink to red. This is of interest because previous investigators have not commented on the nuclear activity of the erythrocyte precursors.

In three cases of multiple myeloma, plasma cells and myeloma cells contained ASA. The cytoplasm of these cells possessed a moderate amount of arylsulfatase activity while the nuclei exhibited an irregular

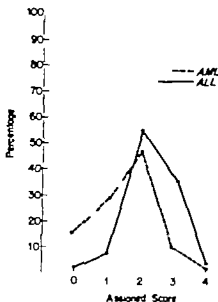


Fig 3. Distribution of scores in the blast forms of acute lymphatic leukemia (ALL) and acute myelogenous leukemia (AML)

arylsulfatase distribution. The areas of arylsulfatase activity appeared to correspond to the areas of parachromatin noted in the WRIGHT's stained smears. Nucleoli were positive

A study was made of a non malignant lymph node imprint to insure the positivity of lymphocytes. The cells showed an intensely positive reaction indicating that arylsulfatase definitely is found in the nucleus of the lymphocyte. The background material of the imprint also appeared to have slight ASA.

In order to evaluate the acute leukemias in terms of quantitative data a scoring system was established in which all blasts were graded on a 0–4+ scale according to the nuclear arylsulfatase activity Difficulty was encountered in distinguishing myeloblasts and lymphoblasts from other round nucleated cells. Nonetheless, an attempt was made to count at least 100 blasts from each smear and grade each of them from 0 to 4 and then calculate the total score by addition Four represented a deep red nucleus 3 a deep pink to bright pink, 2 a pale pink 1 was faint gray (no pink color) A zero score represented no nuclear staining The ability to distinguish the blasts of the leukemic process was facilitated by comparison of arylsulfatase stained smears with WRIGHT's stained smears. Scores between 200 and 220 were

present in both myelogenous and lymphatic leukemias. Assuming the data accumulated to be valid this must be considered the indeterminant range wherein scores cannot be considered of aid in distinguishing the two primary form sof acute leukemia. All scores above 220 were acute lymphatic leukemias and all the scores below 200 were acute myelogenous leukemia. It should also be noted that when the percentage of cells assigned each score in each disease is plotted on a graph (fig 3) scores of 0 and 1 were more frequently assigned to the myelogenous leukemias and scores of 3 and 4 were more frequently assigned to the cells of lymphatic leukemia

Comment

In using this method, it was found that heparin and EDTA had no inhibitory effect on ASA. The time the smears are stored in the desiccator at 4 C is not critical. Slides kept at room temperature for 24 h *and then placed in the 4 C desiccator still show maximum ASA.* It was also observed that activity is still present in smears that have been stored at 4 C for 76 days. ASA can be seen in submaximal amounts after staining for 24 h instead of the specified 48 h

Fifty leukemic patients on various therapies were studied The drugs administered included myleran hydroxyurea prednisone, cytosine arabinoside, vincristine, 6-mercaptopurine, delatestryl, daunomycin, methotrexate and cyclophosphamide Non of the above appeared to effect the ASA.

Summary

A study was made of the nuclear arylsulfatase activity of the cells in various hematologic malignancies. The acute leukemias were investigated by grading the blasts according to arylsulfatase activity on scale of zero to four It was found that lymphatic leukemias generally score higher than myelogenous leukemias. However there is range in which scores from each disease are found, therefore making the stain possibly helpful in differential diagnosis but not inconclusively valid.

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Cytochemical Demonstration of the Co-Enzyme Ubiquinone in Normal Human Blood and Bone Marrow Cells

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The presence of ubiquinone in animal tissue sections of different organs, was first demonstrated by Tranzer and Pearse [7] Hernández and Martinez de Morentin [4] using the same method, demonstrated the co-enzyme cytochemically in all types of peripheral blood cells without giving details of evaluation In the present investigation blood smears from 25 healthy people and 10 samples of morphologically normal bone marrow were examined. We modified the above mentioned method by the use of a suitable fixation technique, a different counter stain and by a semiquantitative procedure of evaluation

Methods

Peripheral blood smears taken from the fingers of healthy people were stained cytochemically duplicates being stained with May-Grunwald-Giemsa stain. Bone marrow was taken from hospital patients either by sternal or iliac crest puncture. They were examined by phase contrast microscopy and were also stained by May-Grünwald-Giemsa stain. Patients diagnoses are detailed in table I

Smears were air-dried for half an hour fixed in formalin vapour for 4 ½ min, rinsed for 5 min in tap water air dried again and incubated for 40 min 37°C in the medium detailed in table II. Control incubations were carried out with the same medium, the hydroquinone being replaced by an equal volume of distilled water After the incubation, the action of catalase was stopped by further exposure to formalin vapour (5 min) One percent aqueous safranin 0 which stains nuclei red orange was used as the counter stain (10 min) The smears were mounted in glycerine jelly (basic)

Ubiquinone concentrations were graded from 1+ to 4+ on the basis of appearance of dark granules, these being classified as fine, medium or coarse. A medium sized granule was held equivalent to 2 fine ones and coarse granule was equivalent to 2 medium or 4 fine ones. The grades given were as follows 1+ = Fine granules only or up to 10 medium granules, 2+ = 10–20 medium granules, 3+ = 20–30 medium granules, 4+ = above 30 medium granules.

Table I. Diagnoses of bone marrow donors

1	Anaemia due to iron deficiency
2.	Pyrexia of unknown origin
3.	Abdominal pain. Diarrhea of unknown origin
4	Anaemia due to iron deficiency. Infectious hepatitis
5.	Toxoplasmosis
6.	Giant follicular lymphoma
7	Lymphadenopathy splenomegaly of unknown origin
8.	Chronic prostatitis. Recurrent urinary tract infection
9	Nephrotic syndrome
10.	Anaemia due to iron deficiency

Table II. Incubation mixture

1	0.50 ml of 0.2 M tris buffer pH 7.4
2.	0.05 ml of 0.5 M cobalt chloride
3.	0.25 ml of MTT 1 mg/ml in distilled water
4	0.10 ml of Catalase crystal suspension 20 mg/ml
5.	0.10 ml of hydroquinone, 40 mg/ml in tris buffer pH 7.4

MTT 3-(4,5-dimethylthiazolyl-2)-5-diphenyl tetrazolium bromide. Sigma Chemical Co., St. Louis 18, Mo.
Catalase C.F. Boehringer & Söhne, GmbH, Mannheim.

Hundred neutrophil granulocytes and 50 lymphocytes were examined in each peripheral blood smear. Monocytes and eosinophils were examined as they occurred. Thrombocytes and erythrocytes were also observed. In bone marrow samples 200 erythropoietic cells, 100 immature granulocytes and 100 mature granulocytes were examined. Plasma cells and megakaryocytes were examined as they occurred. The average grading for each type of cell was calculated from all smears.

In both blood and bone marrow smears incubated in the control medium, no black granules were seen, the reaction therefore being negative.

Results

Peripheral blood. Neutrophil granulocytes were recognized by their large diameter and their lobulated nuclei. Ubiquinone concentrations were lower here than in other cells, their average grading being a little over 1+ (fig. 1a). Eosinophils were recognized by their spectacle-shaped nucleus. In all, a total of 68 eosinophils were examined. Their cytoplasm was packed with dark granules, their grading being close on 4+ (fig. 1b).

Lymphocytes were recognized by their small size and their round and relatively large nucleus which almost filled the cytoplasm. Their grading was close on 2+ (fig. 1a). Monocytes had a conspicuously

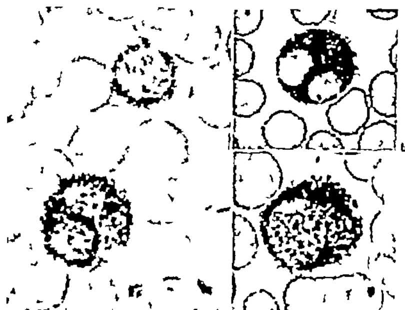


Fig 1 Ubiquinone in peripheral blood cells. Neutrophil granulocyte (below) and lymphocyte (above) *b* eosinophil granulocyte, *c* monocyte (× 1000)

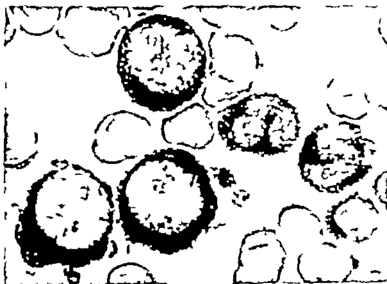


Fig 2. Ubiquinone in bone marrow cells 3 pronormoblasts (at left), one young normoblast (at right below) 2 granulocytes (at right at the middle) (× 1000)



Fig. 3. Ubiquinone in bone marrow cells 3 young granulocytes (above and at right) one young monoblast (in the middle) plasma cell (below) (× 1000)



Fig 4 Ubiquinone in megakaryocyte (1000)

reaction was tried after freezing at $-30°C$, fixation in methanol or in formalin vapour. Best results were achieved with fixation for 4½ min in formalin vapour. Methanol is an unsuitable fixative because it destroys the co-enzyme [1]. Fixation is obviously advantageous as it retains the morphology of the cells intact and also enables smears to be stored till staining is convenient.

Liquid formalin was found to be too drastic, therefore formalin vapour was used to stop the action of catalase. Counterstaining with 2% chloroform extracted aqueous methyl green, as described by HERNÁNDEZ *et al.* [4] was found unsuitable because nuclei stained very faintly and cell identification was therefore difficult. We tried counterstaining with neutral red, neutral fast red (Kernechtrot), Harris haematoxylin and safranin O. The latter gave the most satisfactory results.

Summary

Ubiquinone concentrations were tested cytochemically in 25 blood samples from healthy people and 10 samples of morphologically normal bone marrow. The smears were fixed in formalin vapour, stained cytochemically and counterstained in safranin O. The presence of ubiquinone was demonstrated in all cells. Highest concentrations were found in eosinophil granulocytes and monocytes of peripheral blood and in plasma cells and megakaryocytes of bone marrow. Ubiquinone in the erythropoietic and granulocytopoietic systems progressively decreased with the maturity of the cell.

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The Comparison of the Coagulation Factors in Arterial and Venous Blood

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The coagulation mechanism has been considered in a state of dynamic equilibrium with a limited amount of clotting occurring continually and excessive accumulation of fibrin being prevented by fibrinolysis [1]. If this were so, because of sluggish circulation and liability to stagnation, it might be expected that blood coagulation would proceed more rapidly in the venous system than on the arterial side. It would be logical to expect that some clotting factors, such as factors II, V and VIII and fibrinogen, which are used during coagulation, would be less in peripheral venous blood than in the arterial blood. In addition to this, since most of the coagulation factors are synthesized in the liver, higher activities of these factors should be found in the arterial blood.

This study was conducted to test this hypothesis by comparing the coagulation factors in the arterial and venous blood because this sort of investigation has rarely been performed [2, 3].

Materials and Methods

The coagulation factors were assayed simultaneously in the arterial and venous blood of 18 persons, 14 males and 4 females, aged 12 to 23 years. Fourteen of these were either laboratory personnel or medical students. Their blood samples were obtained from the antecubital vein of one arm and the brachial artery of the other arm with 2 or 3 min interval. In 4 patients blood samples were obtained at the beginning of cardiac catheterization, prior to heparinization. None of these 4 were cyanotic or in distress. (One patient had an atrial septal defect and the other 3 ventricular septal defects.) Blood samples were also obtained from the antecubital vein and brachial artery in the catheterized patients. In all cases the venous blood was obtained first. If any difficulty was encountered in either arterial or venous

large, broad and indented nucleus. Their ubiquinone concentrations were very high the cytoplasm being full of fine medium and coarse granules A total of 160 monocytes were examined in all smears, their grading was close on 4+ (fig 1c)

Thrombocytes generally occurred in aggregates. The granules seen in these cells were generally fine and medium in size, their grading being between 1+ and 2+ Erythrocytes also contained black granules, these were generally fine and situated at the periphery of the cell.

Bone marrow Pronormoblasts were found to be large cells with a round nucleus having well defined outlines. The cytoplasm contained black granules of all sizes, their grading varying from 2+ to 3+ (fig 2) Young normoblasts were smaller than pronormoblasts, their nuclei were well defined and were surrounded by a medium sized cytoplasm. The concentration of ubiquinone was lower than in pronormoblast generally close on 2+ (fig 2 and 3) Mature normoblasts were still smaller their nuclei were round and well defined, the cytoplasm being relatively broad. The concentration of ubiquinone was lower than in young normoblasts being 1+

Young granulocytes were equal in size or smaller than pronormoblasts. Their nuclei were large, slightly indented their outlines were not so well defined. The coenzyme concentration in these cells was close on 3+ (fig 3) Mature granulocytes were morphologically similar to those found in peripheral blood, their grading was a little above 1+ (fig 2)

Megakaryocytes were recognizeable by their giant size They contained a large number of black granules generally small and medium sized with occasional coarse granules occurring A total of 22 megakaryocytes were examined in all samples. Their grading was 4+ (fig 4)

The cytoplasm in plasma cells was broad the nucleus was relatively small and generally eccentric. Their grading was very high, reaching 4+ (fig 3) many coarse black granules being found in the cytoplasm. A total of 87 plasma cells were examined Ubiquinone concentrations in peripheral blood and bone marrow cells are summarized in figure 5.

Discussion

Ubiquinone (co-enzyme Q) is a co-enzyme acting as an electron transfer agent between flavoproteins and the cytochrome system in

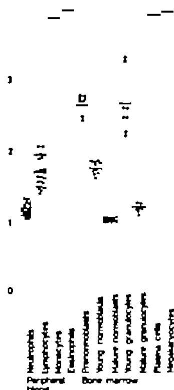


Fig. 5. Ubiquinone concentrations in peripheral blood and bone marrow cells. Each point represents the average in single sample. Due to small number of cells found only the general average is given.

the intracellular respiration process. Furthermore it seems likely that it participates in energy coupling (oxidative phosphorylation) [2, 5 6] This co-enzyme is insoluble in water and is found especially in the cristae and internal membranes of the mitochondria [3]

The cytochemical demonstration of ubiquinone is based on differences between the redox potential of hydroquinone ubiquinone and tetrazoluim salt, enabling the specific electron transfer from hydroquinone to tetrazolium salt through ubiquinone [7] The reduced tetrazolium salt formazan is precipitated in shape of black granules.

Hernández and Martinez de Morentin [14] performed the cytochemical staining without fixation. In this investigation, the

reaction was tried after freezing at − 30 C, fixation in methanol or in formalin vapour Best results were achieved with fixation for 4½ min in formalin vapour Methanol is an unsuitable fixative because it destroys the co-enzyme [1] Fixation is obviously advantageous as it retains the morphology of the cells intact and also enables smears to be stored till staining is convenient.

Liquid formalin was found to be too drastic, therefore formalin vapour was used to stop the action of catalase. Counterstaining with 2% chloroform extracted aqueous methyl green, as described by HERNÁNDEZ *et al.* [4] was found unsuitable because nuclei stained very faintly and cell identification was therefore difficult. We tried counterstaining with neutral red, neutral fast red (Kernechtrot) Harris haematoxylin and safranin 0 The latter gave the most satisfactory results.

Summary

Ubiquinone concentrations were tested cytochemically in 25 blood samples from healthy people and 10 samples of morphologically normal bone marrow The smears were fixed in formalin vapour stained cytochemically and counterstained in safranin 0. The presence of ubiquinone was demonstrated in all cells. Highest concentrations were found in eosinophil granulocytes and monocytes of peripheral blood and in plasma cells and megakaryocytes of bone marrow Ubiquinone in the erythropoietic and granulocypoietic systems progressively decreased with the maturity of the cell.

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The Comparison of the Coagulation Factors in Arterial and Venous Blood

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The coagulation mechanism has been considered in a state of dynamic equilibrium, with a limited amount of clotting occurring continually and excessive accumulation of fibrin being prevented by fibrinolysis [1]. If this were so, because of sluggish circulation and liability to stagnation, it might be expected that blood coagulation would proceed more rapidly in the venous system than on the arterial side. It would be logical to expect that some clotting factors, such as factors II, V and VIII and fibrinogen, which are used during coagulation, would be less in peripheral venous blood than in the arterial blood. In addition to this, since most of the coagulation factors are synthesized in the liver, higher activities of these factors should be found in the arterial blood.

This study was conducted to test this hypothesis by comparing the coagulation factors in the arterial and venous blood because this sort of investigation has rarely been performed [2, 3].

Materials and Methods

The coagulation factors were assayed simultaneously in the arterial and venous blood of 18 persons, 14 males and 4 females, aged 12 to 23 years. Fourteen of these were either laboratory personnel or medical students. Their blood samples were obtained from the antecubital vein of one arm and the brachial artery of the other arm with 2 or 3 min interval. In 4 patients blood samples were obtained at the beginning of cardiac catheterization, prior to heparinization. None of these 4 were cyanotic or in distress. (One patient had an atrial septal defect and the other 3 ventricular septal defects.) Blood samples were also obtained from the antecubital vein and brachial artery in the catheterized patients. In all cases the venous blood was obtained first. If any difficulty was encountered in either arterial or venous

puncture, the blood samples were discarded. With the exception of catheterized patients, venous tourniquet was applied for only short period of time to allow insertion of the needle.

In the present investigation the following coagulation tests were used prothrombin time [4] partial thromboplastin time [5] assays of prothrombin, factor V and factors VII/X combined activity [6]; fibrin stabilizing factor [7]; assay of factor VIII [8]; fibrinogen [9]; platelet count [10] and hematocrit determined by the microhematocrit method.

Results

Most of the results are summarized in table I In 12 cases the fibrin stabilizing factor and in 8 cases the clot retraction were the same in arterial and venous blood In 16 cases the capillary platelet count was slightly higher than in arterial and venous blood however the difference was not statistically significant.

In all of these determinations only factor VIII and fibrinogen were significantly lower in venous than in arterial blood ($P < 0.05$ for both) (table II)

Discussion

In most previous studies, arterial and venous blood were compared for fibrinolytic activity Although there is some controversy in general this activity was found higher in venous than in arterial blood [2, 11 12]

In normal pigs prothrombin time, platelet count platelet adhesiveness index, clotting time and thromboplastin generation time have been determined in arterial and venous blood. Only the clotting time was found significantly shorter in venous than in arterial blood [3].

In normal human subjects the arteriovenous differences of blood coagulation factors were examined by Nanu *et al* [2] They showed a trend toward lower coagulation activity in the arterial blood as indicated by Quick prothrombin time. They have also determined Stypven time, prothrombin, factor V factor VII/X, heparin tolerance and thromboplastin generation with inosithin The arteriovenous differences, however were not all in the same direction some indicated increased and others decreased coagulation activity of the arterial blood.

In the present study Quick prothrombin time and *PTT* were *not* statistically different in arterial and venous blood however they were

Table I Coagulation factors in arterial and venous blood

Tests		Arterial blood				Venous blood		
		range	mean	S.D		range	mean	S.D.
Quick time sec	6	11.3–19	14.2 ± 1	2.5	6	11.2 17.0	14 7 ± 0.8	2
Partial thromboplastin time sec	6	51 100	82.1 ± 8.2	20	6	48–122	83 ± 9 1	22 1
Prothrombin, %	17	50–127	86.5 ± 5.1	21.3	18	46–162	91.8 ± 8	34 4
Factor V %	17	55–158	96.2 ± 7 1	29.7	17	56–100	103.5 ± 8.5	35.2
Factor VII/X, %	16	63–107	120.2 ± 10.4	41.9	16	71 285	113 6 ± 13.8	55.3
Factor VIII %	11	53–228	112.3 ± 14.9	49.6	11	44–196	98.6 ± 13.3	44.3
Fibrinogen, mg %	12	257–423	338.7 ± 13.4	46.5	12	226–400	311.2 ± 16.7	.8
Hematocrit, %	12	35–46	39.5 ± 1	3 6	10	33.5–43	38.9 ± 0.8	2.8
Platelets, $10^3/mm^3$	18	81 248	165 ± 8.2	35.2	16	76–214	153 4 ± 8 9	35.9

– number of samples on which determinations were performed

Table II. The factor VIII and fibrinogen levels of arterial and venous blood in paired samples

	Antihemophilic globulin % (11 subjects)			Fibrinogen mg % (12 subjects)	
	arterial	venous		arterial	venous
	70	60		354	354
	146	85		283	270
	116	118		257	257
	114	98		301	308
	115	133		298	226
	72	48		322	296
	150	140		322	314
	228	196		377	400
	53	48		380	277
	117	105		330	255
	55	44		423	378
	-	-		400	400
Mean	112.3	98.6	Mean	338.7	311.2
	±14.9	±13.5		±13.4	±16.7
S.D	49.6	44.5	S.D.	46.5	58.0
t	2.54		t	2.229	
P	<0.05		P	<0.05	

determined in only 6 cases. Since most of the clotting factors were assayed one by one, it was not thought that the slight changes would be reflected in these relatively crude determinations. In this study prothrombin factor V and factor VII/X assays revealed slightly higher yields in the arterial blood in contrast to the findings of NADM *et al.* [2] However these changes were not statistically significant in either study

We have also compared fibrinogen and factor VIII levels in the arterial and venous blood in 11 and 12 paired samples (table II)

Statistically significant decreases were found for both in the venous blood when the means of the individual differences found between the values observed in each pair of samples were examined ($P < 0.05$) As GARDIAKIS *et al* [13] found that factor VIII concentration of the hepatic venous blood is higher than in peripheral venous blood, this result can be explained. They found that the concentration of factor VIII in the plasma of the blood of the hepatic vein was consistently and significantly higher than that of the venous blood (+150) The

magnitude of the difference between the hepatic and the peripheral venous blood might not be reflected between arterial and venous blood, because of the dilution of hepatic blood in the arterial system. Although the liver may not be the only organ involved it is the most important site for fibrinogen and factor VIII synthesis. For that reason, increased fibrinogen level in the arterial blood, like factor VIII would not be a surprising finding

The lower levels of factor VIII fibrinogen in the venous blood might suggest a continuous clotting process in the periphery With this supposition, the low level of factor V and to some degree of prothrombin, would be expected. But this was not the case. Although it is still a possibility that continuous blood clotting takes place in the capillaries, our findings do not fully support this.

Our platelet counts in arterial and venous blood were generally much lower than would be expected by the REES-ECKER technique. This was most likely due to the handling of the specimen in the non-siliconized glass tubes, but our capillary platelet count values were also much lower than for this method Since the samples from both sites were obtained under the same conditions the arterial and venous platelet counts can be compared This kind of difference between arterial and venous blood has also been published previously for animals [8] and for human beings [14] However the difference is not statistically significant.

In this study an insignificant difference between arterial and venous blood was also observed in the hematocrit values. Since hematocrit values in venous blood are slightly lower the differences in the platelet counts might be due to the dilution effect.

From this study it might be concluded that there is no statistically significant difference of coagulation factors between arterial and venous blood to support continuous blood clotting in the capillaries.

Acknowledgment. I am indebted to Dr TAHSIN TUNCALI for obtaining blood from normal and catheterized patients, and to Mr METIN A ASU for the statistical analysis.

Summary

Comparative studies of coagulation factors in arterial and venous blood were made in 14 normal subjects and in 4 patients with atrial or ventricular septal defect. No difference was

found in the level of prothrombin, factor V factor VII/X, retraction, factor XIII and platelet count. Fibrinogen and factor VIII were significantly low in the venous blood. The platelet count was higher in the capillaries than in the arterial and venous blood.

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A Case of IgAλ Myelomatosis with Two Urinary Bence-Jones Proteins (BJλ and BJL) and Multiple Chromosomal Abnormalities

F. Dammacco, D. Trizio and L. Bonomo

In sera from patients with multiple myeloma the presence of two different paraproteins or M-components is rather rare. The most frequent event is a combination of IgG- and IgA-components [1, 7, 12, 18, 24], but the association of a single paraprotein in the serum and of 2 Bence Jones proteins in the urine has been reported, to our knowledge, only once [13].

It is the purpose of the present report to describe a case of IgA myelomatosis, in which 2 immunologically different Bence Jones proteins were detected in the urine. Multiple chromosomal abnormalities were a further interesting aspect of this case.

Case Report

L.V., a 69-year-old worker from Bari, Italy, was transferred to our wards from a hospital of a small town near Bari. At the age of 18 the patient suffered from malaria, which disappeared 5 months later by quinine therapy. He was then in apparently good health until 51, when he experienced several episodes of urolithiasis and complained of dysuria and stranguria. One year later he was operated on for right inguinal hernia and lithiasis of the urinary bladder at the same time. The bladder was reported to appear chronically inflamed and containing numerous calculi; the dysuric symptomatology did not, however, subside completely after the operation.

One month prior to the present admission the patient experienced again recurrent attacks of abdominal pain, which irradiated from the right lumbar region forward and down to the corresponding testis. He was admitted to the surgical department of a hospital in the province of Bari, where clinical, radiological and laboratory findings were consistent with the diagnosis of lithiasis of the right kidney. The increased values of the blood urea nitrogen

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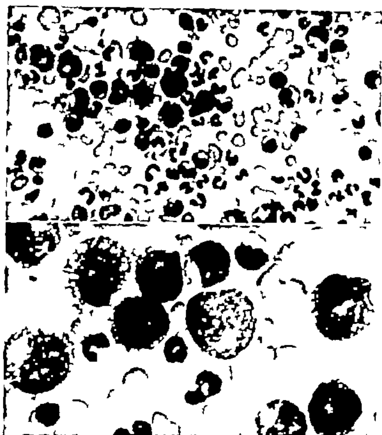


Fig. 1. Bone marrow smears. Nest-like infiltration of plasma cells (440). *b* At higher magnification (1,000) the 'thesaurocyte' appearance of some plasma cells is clearly evident.

(180 mg%) did not allow any surgical treatment and, on the other hand, an unexpected marked leukocytosis (70,000 white blood cells/mm) raised the problem of coexistent blood disease. The patient was, therefore transferred to the medical section of the same hospital. Extreme fatigue, anemia and moderate splenomegaly were recorded erythrocyte sedimentation rate and blood urea nitrogen were remarkably increased. Electrophoresis of the serum proteins was not performed.

The patient was treated with fluids, vitamins, etc. for 2 weeks, and then entered our hospital for further studies. Fatigue, weight loss, anorexia, nausea and vomiting were the chief complaints on admission. Physical examination disclosed chronically ill, pale subject. There was moderate hepatosplenomegaly and small axillary lymph nodes were palpable. Scattered coarse rales were present at the posterior bases of both lungs.

Blood pressure 195/110 mm Hg. Haemoglobin 7.2 g% (49% RBC 2.5 million/mm³ (overlapping values were found on subsequent controls). WBC 63,000 mm³ neutrophils 92%, lymphocytes 7%, monocytes 1) on admission, 56,000 mm³ after one week of cyto-

static treatment, and 40,500/mm after 10 days of therapy. The neutrophilia persistently ranged from 84 to 92%; no immature cells were seen in the peripheral smears, but 1–2 plasma cells/500 nucleated elements were found in different controls. ESR 117 mm/1 h (subsequently 74 mm/1 h). Fasting blood glucose 100 mg%. Blood urea nitrogen 169–375 mg%. Serum sodium and potassium levels 310 and 30 mg%, respectively. Electrocardiogram showed the signs of left ventricular hypertrophy. Urinalysis: specific gravity 1,006–1,010; protein traces were repeatedly present; the sediment was crowded with leukocytes mostly clumped and degenerated, and several erythrocytes. Bacteriological examination of the urine was not performed.

Paper electrophoresis of serum proteins: albumin 46.3, α_1-globulin 2.5, α_2-globulin 5.7, β-globulin 6.2, γ-globulin 39.0°; albumin/globulin ratio 0.71. Total serum protein (biuret method) 7.0 g%. A homogeneous band, giving a monoclonal-type spike, was clearly recognizable in the β-γ-area: it accounted for 30% of serum proteins (2.1 g%).

Chest roentgenogram showed accentuated broncho-vascular markings and slight left ventricular prominence. Abdominal plain roentgenogram revealed a stamp-like calculosis, occupying completely the right pelvis and calices, whereas the left kidney appeared enlarged. Skeletal X-ray survey (skull, ribs, thoracic and lumbar spine, pelvis and proximal humera and femura) *disclosed osteoporosis of the lumbar spine, much more marked than it would* be expected solely on the basis of the patient's age, and with a characteristic spotty disposition; moreover, very few osteolytic regions were seen on the right femur. Bone marrow aspirate (fig. 1) showed that the granuloblastic series was present in all phases of maturation. Twenty per cent of the nucleated elements were plasma cells, and many of them had monstrous appearance, with pycnotic nuclei and numerous compartments in their cytoplasm ('thesaurocytes' according to PARASKEVAS *et al.* [22]). The erythroblastic series was rather scanty.

The patient was treated with cyclophosphamide (50 mg daily), prednisone (25 mg daily), fluids, etc. with initial improvement of the subjective symptomatology. The blood urea nitrogen, however, rose progressively to 375 mg%, and insisting vomiting and belching appeared. On his demand, the patient was discharged and followed at home by one of us (F.D.). His general condition deteriorated rapidly until uraemic coma and death supervened. Routine examinations performed 2 days before death did not show any remarkable changes as compared with previous results. Autopsy was not allowed.

Special Studies

Agar-gel-electrophoresis and *immunoelectrophoresis* were performed as described elsewhere [8]. Polyvalent horse immune serum against normal human serum and strictly specific anti-IgG, anti-IgA and anti-IgM rabbit antisera were obtained from the Netherlands Red Cross Blood Transfusion Service, Amsterdam. Anti-κ and anti-λ rabbit antisera were prepared in our laboratory.

The serum agar-electrophoresis (fig. 2) showed a prominent, discrete component with β_1-mobility and, more cathodically, an additional faint spike in γ-position. By immunoelectrophoresis the main M-component was identified as belonging to the IgA class (fig. 3a, b) with κ-type light chains (fig. 4). The other rudimentary component could not be characterized *with certainty owing to its faintness, but was identified as κ-type Bence-Jones protein* by comparison with the urine electrophoretic and immunoelectrophoretic patterns (see below). Serum immunoglobulin levels (simple radial immunodiffusion) per 100 ml: IgG 600 mg; IgA 2,240 mg; IgM 30 mg.

The heat test for Bence-Jones proteinuria gave no clear-cut results because of coexistent albuminuria. Agar-electrophoresis of 50-fold concentrated urine disclosed, along with albumin and traces of α_1- and α_2-globulins, 3 discrete components (fig. 2). Proceeding from the



Fig. 2. Agar-gel electrophoresis of the patient serum (S) and urine (U). In the serum along with the main M-component, a faint monoclonal band is visible in a more cathodal position. The urinary pattern shows 3 discrete components: the first component (from left to right) corresponds for its mobility to the serum main paraprotein, and is likely due to traces of this serum component leaked into the urine; the second component, quantitatively the most important, shows a mobility corresponding to the serum additional band and was interpreted as a Bence-Jones protein; finally, the third most cathodal component has no serum counterpart.

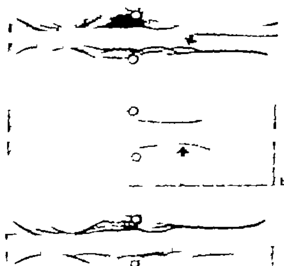


Fig. 3. Immunoelectrophoresis of the patient serum and urine. *a* Pooled normal human serum (upper well) and patient' serum (lower well); polyvalent anti-human antiserum in the through. *b* Upper and lower wells as in *a*. Specific anti-IgA antiserum in the through. The abnormal arc visible in *a* is identified in *b* as IgA-type paraprotein. *c* Pooled normal human serum (upper well) and 50-fold concentrated urine (lower well). Polyvalent anti-human antiserum in the through. Note the unselective proteinuria, determining the passage of many serum protein fractions into the urine.

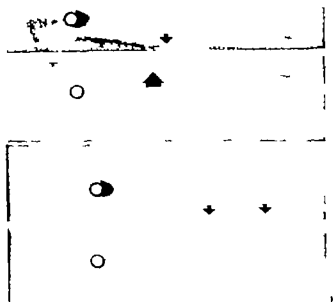


Fig 4 Immunoelectrophoresis of urine (upper wells) and serum (lower wells) Pattern obtained with specific anti-κ antiserum. The serum IgA-paraprotein and the Bence Jones protein giving the main urinary component are identified as belonging to the immunologic κ-type. *b* Pattern obtained with specific anti-λ antiserum. A second, faint, λ-type Bence-Jones protein is recognizable in the urine.

anodal to the cathodal end, the first component had the same mobility as the serum main spike and was presumably due to the leakage into the urine of traces of whole IgA. The second component, which was quantitatively the most important, possessed mobility comparable to the serum additional spike, and was interpreted as being Bence Jones protein. Finally the third discrete component had no detectable serum counterpart and could be ascribed to second Bence Jones protein by further studies (see below)

Immunoelectrophoresis of the urinary proteins (fig 3c) confirmed the presence of albumin and small amounts of γ-lipoproteins, α_1- and β_1-globulins; traces of IgA and IgG *were detected as well. A thick precipitation arc was revealed by the specific anti-κ antiserum* (fig. 4a) thus identifying the main urinary component as κ-type light chains. Furthermore, another precipitation arc, rather weak but clearly recognizable in the cathodal region (fig. 4b), was obtained in the urine with specific anti-λ antiserum, suggesting that the urinary component seen in the most cathodal position by agar-electrophoresis was indeed second λ-type Bence-Jones protein.

A direct confirmation to the existence of the urinary biclonal gammapathy was achieved by separation of the urinary proteins on Sephadex G-150 column (50 x 3 cm) using 0.1 M phosphate buffered saline as the eluent. The fractions corresponding to the descending half of the last chromatographic peak were pooled and concentrated. When tested by immuno-

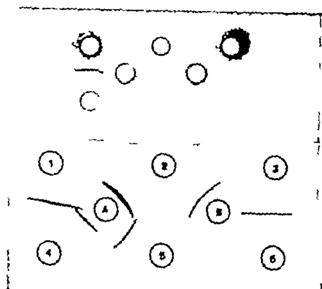


Fig. 5. Ouchterlony gel double diffusion test. The patient's urinary proteins were separated on Sephadex G-150 column, and the fractions corresponding to the descending half of the last chromatographic peak were pooled, concentrated and tested in this system. *1* Horse anti-human IgG antiserum, *2* concentrated urinary fractions. *3* horse anti-human IgA antiserum. *4* IgGλ-myeloma protein, isolated from a patient with myelomatosis. *5* pooled normal human sera. *6* IgAλ-myeloma protein, isolated from patient with myelomatosis. *A* Rabbit anti-κ chain antiserum. *B* rabbit anti-λ chain antiserum. The urinary proteins of well 2 do not react against the anti-IgG and anti-IgA antisera, but clearly give precipitation line with both anti-κ and anti-λ antisera.

diffusion against anti-IgG, anti-IgA, anti-κ and anti-λ specific antisera, the concentrated sample *did not show detectable amounts of IgG and IgA, but did reveal* κ *and, to* a lesser extent, λ-type light chains (fig. 5).

Total urinary protein was determined by the biuret method on the redissolved precipitate from 20% trichloroacetic acid. By scanning the urinary electrophoretic pattern, it was then possible to calculate the approximate amount of Bence Jones proteinuria. The level of *κ-type light chains was found to be 1.3 g/l and that of λ-type light chains resulted* 0.3 g/l. Therefore, the calculated total amount of Bence-Jones protein was approximately 1.6 g/l.

Analytical ultracentrifugation of the serum proteins, carried out in a Beckmann Spinco Model E apparatus, was non-contributory. The 19S globulins were hardly visible and the serum main M-component, corresponding to the ultracentrifugal G peak, had a sedimentation constant of 7.5 S and accounted for 32% of the total protein.

Immunofluorescence studies on bone marrow smears were performed as described previously [5] using fluorescein-isothiocyanate-labelled anti-human IgA and IgG rabbit antisera. A great number of plasma cells appeared strongly stained by the fluoresceinated anti-IgA antiserum, suggesting they were actively synthesizing the IgA paraprotein; sometimes the fluorescence showed a characteristic vacuolar distribution (fig. 6). A definitely smaller number of plasma cells stained with the fluoresceinated anti-IgG antiserum, and the ratio

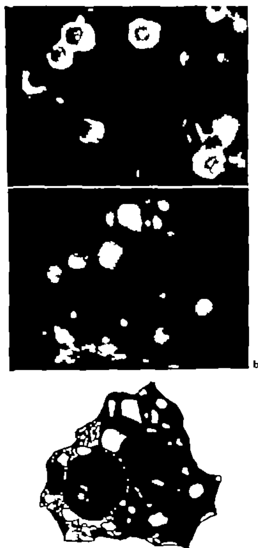


Fig. 6 Immunofluorescence pattern of bone marrow smears, stained with fluorescein-isothiocyanate-labelled anti-IgA antiserum. Several plasma cells show strong fluorescence (original magnification 250) *b* Typical 'thesaurocyte' with mesh-like fluorescent staining (original magnification 1 140) Schematic drawing of the plasma cell represented in *b*

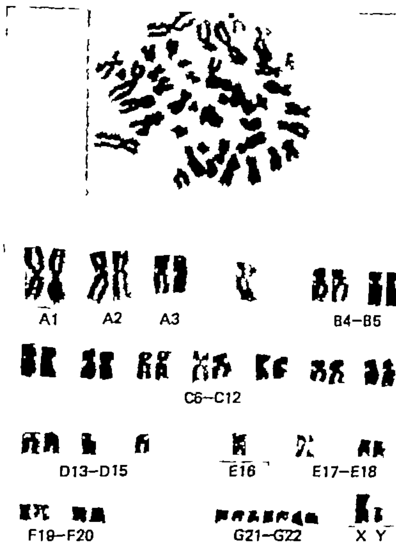


Fig. 7. Abnormal karyotype from a bone marrow metaphase plate with 46 chromosomes. Note one extrachromosome in the AB size range (MG-chromosome); 2 chromosomes in D and 2 in E series are missing; 3 extra chromosomes in the G series.

of IgA/IgG containing cells was approximately 5. The fluorescence determined by one antiserum could be blocked by the corresponding unconjugated antiserum, without affecting the fluorescent staining produced by the other antiserum.

Chromosome studies were performed on direct bone-marrow air-dried preparations by the method of Tjio and Whang [25]. Peripheral blood cultures were used as well, following the method of Moorhead *et al.* [20] with minor modifications. Neither the time of analysis

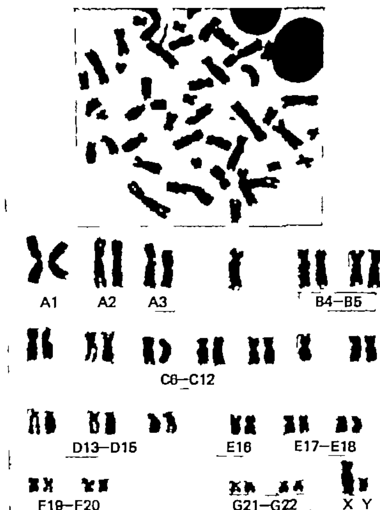


Fig. 7. Pseudodiploid karyotype from peripheral blood culture metaphase plate. An MG-chromosome is visible. chromosome of the C group is missing. Note chromatid breaks in A3 and in one of the C group.

nor previously the patient had been treated by chemotherapy, radiation or corticosteroids. The combined Denver [11] and PATAU [23] system of nomenclature is used.

Chromosome counts of marrow and peripheral blood cells are given in table I. Twenty one cells were counted and karyotyped on bone marrow, and only 2 abnormal cells were observed. One with two chromosomes in the G series missing, the other pseudodiploid with very profound disturbance of the karyotype (fig. 7), namely one extra chromosome

Table I. Chromosome counts of bone marrow and peripheral blood cells

	Cells counted	Number of chromosomes			
		44	45	46	47
Bone marrow	21	1	–	20 (1)[1]	
Peripheral blood	52	3	26	14 (10)	9

[1] The number of pseudodiploid cells in parentheses.

in the AB size range (MG-chromosome according to Houston *et al.* [17]), 2 chromosomes of the D and 2 of the E series missing, and finally 3 extra chromosomes resembling those of the G series. In contrast to the results obtained on peripheral blood (see below), the relatively poor percentage of abnormal karyotypes in bone marrow cells is likely due to the fact that *the great majority of the cells in mitosis* (observed on routine May-Grünwald-Giemsa-stained bone marrow smears) were of the myeloid series.

In peripheral blood cultures 52 metaphase plates were counted and analysed (table I) and only 4 of them had 46 chromosomes with normal male karyotype. There was an abnormal incidence of chromatid gaps and breaks (fig. 8). The aneuploid and pseudodiploid cells showed a remarkable number of abnormalities, without uniformity and with a variety of chromosomal rearrangements in all groups. In hypodiploid cells with 45 chromosomes the most common feature (9 cells) was monosomy of G 21 or G 22. Two other cells of this group showed one MG-chromosome with two G chromosomes missing. In the pseudodiploid *group 4 cells showed one MG-chromosome with lack of one C chromosome (fig. 8).* Finally no consistent pattern could be established in other hypo-, pseudo- and hyperdiploid cells, but 4 other cells with one MG-chromosome were recognized among hyperdiploid cells.

Discussion

The clinical diagnosis in our patient was not entirely clear until after thorough investigation. Indeed, although *post-mortem* examination could not be performed, the demonstration of a serum M-component, decrease of normal serum immunoglobulins, diffuse plasmacytosis with abnormal morphology of most plasma cells, spotty osteoporosis of the lumbar spine, rare osteolytic lesions and presence of Bence Jones proteinuria left little doubt about the diagnosis of multiple myeloma.

A most interesting feature was the urinary diclonal gammapathy. Actually, 2 immunologically different Bence-Jones proteins were unequivocally demonstrated in the urine of this patient, similarly to the case described by Engle and Nachman [13]. The total amount of the urinary light chain excretion (1.6 g/l) was considered as a further support to the diagnosis of myelomatosis since it has been found that

Bence Jones proteinuria in excess of 100 mg/100 ml strongly argues for myelomatosis or primary macroglobulinemia [9 16] Since the 2 urinary Bence Jones proteins were of different immunological type it seems likely that they were synthesized by two genetically distinct cell lines.

The marked leukocytosis was tentatively thought to depend mainly on the coexistent urolithiasis and pyelonephritis. The patient had in fact a history of 18 years duration, consisting in recurrent attacks of urolithiasis, stranguria and dysuria. Although the relationships between multiple myeloma and chronic infections of the biliary tract or of the urinary apparatus are not clearly understood it has been suggested that a chronic reticuloendothelial stimulation might trigger a plasmacytic dyscrasia in genetically-conditioned hosts, acting as an adjuvant like stimulation [21]

A final important point regards the multiple chromosomal abnormalities. Chromosomal aberrations have been reported in Waldenström's macroglobulinemia [2, 5 14 15] but no consistent abnormalities were previously found in multiple myeloma [4 6, 10 19] Recently Houston *et al.* [17] have described the presence of an extra-chromosome common to IgG- IgA and IgM type monoclonal gammapathies (MG-chromosome) In our patient, along with other heterogeneous chromosomal alterations, such MG-chromosomes were indeed detected in hypo- pseudo- and hyperdiploid cells The significance of the MG-chromosomes is still uncertain. Available evidence does not prove they are true marker chromosomes pathognomonic of plasma cell dyscrasias and related conditions. Their demonstration in the 3 types of monoclonal gammapathies lends, however support to the hypothesis that a common, still unknown defect may be responsible for the formation of the MG-chromosomes [17]

Summary

A case of multiple myeloma is described, whose serum contained an IgAK-myeloma protein. Two immunologically different Bence-Jones proteins, one of the κ-type and the other of the λ-type, were demonstrated in the urine of the same patient. Multiple chromosomal abnormalities were also detected in both bone marrow cells and peripheral blood cultures.

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The Effect of Cytosine Arabinoside on Nucleic Acid Synthesis in Normal and Leukaemic Human Leucocytes *in vitro*

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Cytosine arabinoside is a synthetic pyrimidine nucleoside. It inhibits replication of DNA viruses e.g. vaccinia virus [1] and has *in vivo* anti-tumour activity against the L1210 mouse leukaemia and a variety of rodent leukaemias and lymphomas [2, 3, 4]. Preliminary trials showed it to be a promising new agent in the therapy of human acute leukaemia [5] and further clinical evaluation is currently in progress. This paper reports the results of *in vitro* culture studies on the effects of this drug on DNA and RNA metabolism in human leucocytes, including both normal and leukaemic cells.

Materials and Methods

Blood cells were obtained from 4 patients and marrow cells from one patient with acute leukaemia. Their cytological type, clinical status and therapeutic history are shown in table I. None of the patients had been treated with cytosine arabinoside. In patients 2, 3, 4 and 5 the only primitive cells in the peripheral blood were leukaemic blast cells. In patient 1 however, in addition to the leukaemic blast cells (83%) there were 2% promyelocytes and myelocytes and 1.5% normoblasts (the remaining 13.5% consisted of mature polymorphs). Cytologically and cytochemically this case was unquestionably an acute lymphoblastic leukaemia. Since the appearance of myeloid and erythroid precursors in the peripheral blood was a transient phenomenon which occurred during a successful induction of remission, it was considered that these cells were not leukaemic cells but belonged to normal cell lines which were re-emerging in the marrow with overspill into the peripheral blood. This case therefore gave an opportunity to study both leukaemic and normal proliferating cells from the same individual in one culture.

The cells were suspended in culture medium containing 70% TC 199 (Glaxo) and 30% plasma at a leucocyte concentration of 3,000 cells per mm³. Cytosine arabinoside¹ was added

¹ Kindly provided by the US Public Health Service.

Table I. Description of patients studied

Case No.	Cytological and cytochemical type of acute leukaemia	Clinical status	Previous treatment
1	lymphoblastic	relapse	Prednisone Vincristine 6-mercaptopurine
2	lymphoblastic	new case	none
3	lymphoblastic	relapse	Prednisone Vincristine 6-mercaptopurine
4	leukaemic phase (classified as myelo-monocytic) of reticulosarcoma	relapse	Prednisone Vinblastin Cyclophosphamide
5	lymphoblastic	new case	none

at a concentration of 0.5 µg/ml (2×10^{-6}M) a duplicate culture without this drug was kept as a control in all cases. After incubation for 1 h at 37°C, ^{3}H-thymidine[1] 0.5 µCi/ml (specific activity 2 Ci/M) was added to the cultures. In the case of patient 1 a second pair of cultures was set up as above but with uridine-5-^{3}H 1 µCi/ml (specific activity 5 Ci/M) in place of ^{3}H-thymidine.

Normal marrow cells were obtained by sternal puncture from normal individual. Two pairs of cultures (control and with cytosine arabinoside) were set up as above with ^{3}H-thymidine and uridine-5-^{3}H.

All cultures were incubated for 30 min in radioactive medium, then concentrated by centrifugation and smeared on glass slides. Autoradiographs were then prepared using Ilford L4 emulsion, exposed for 6 days and stained with the PAS reaction. Grain counts were then made on 100 cells in each culture. In cultures from patient 1 the various cell types (lymphoblasts, promyelocytes, myelocytes and normoblasts) were easily distinguished because of differences in PAS positivity as well as cytological features.

Results

In the ^{3}H-uridine cultures, no difference was found between the cultures treated with cytosine arabinoside and the controls both as regards the percentage of cells labelled and the intensity of labelling in any of the cell types studied (table II). Thus RNA synthesis was not affected by the presence of the drug in the cultures.

In the ^{3}H thymidine cultures (table III) a difference in the degree of inhibition of DNA synthesis by cytosine arabinoside in different cell types was observed, the inhibition being much more marked in the leukaemic cells. In 4 of the 5 samples of leukaemic cells studied, the percentage of cells labelled in the cultures treated with cytosine

[1] Radiochemical Centre, Amersham, England.

Table II. Results in cytosine arabinoside (Ara-C) treated cultures and controls. Studies with uridine-5-³H

Culture	Percent cells labelled Control	Percent cells labelled Ara-C	Grain counts per 100 cells Control	Grain counts per 100 cells Ara-C
Acute leukaemia, case 1:				
Lymphoblasts	100	99	3 425	3,175
Promyelocytes and myelocytes	98	99	8,108	8,004
Normoblasts	99	97	2,214	2,172
Normal marrow				
Promyelocytes and myelocytes	100	100	6,496	6,620
Normoblasts	99	98	3,388	3,295

Presumed normal cell lines.

Table III. Results in cytosine arabinoside (Ara-C) treated cultures and controls. Studies with ³H-thymidine

Culture	Percent cells labelled Control	Percent cells labelled Ara-C	Grain counts per 100 cells Control	Grain counts per 100 cells Ara-C
Acute leukaemia				
Case 1: Lymphoblasts	19	13	1,963	32
Promyelocytes and myelocytes	74	73	4,720	3,006
Normoblasts	40	36	1 717	637
Case 2 Lymphoblasts	5	0.5	972	10
Case 3 Lymphoblasts	15	0.3	2,950	70
Case 4 Myelomonoblasts	4	0	1,000	0
Case 5 Lymphoblasts	26	0	850	0
Normal marrow				
Promyelocytes and myelocytes	72	69	5,860	2,213
Normoblasts	36	30	2,307	502

Presumed normal cell lines.
Cases 1 2, 3, 4 peripheral blood cultures. Case 5 marrow cultures.

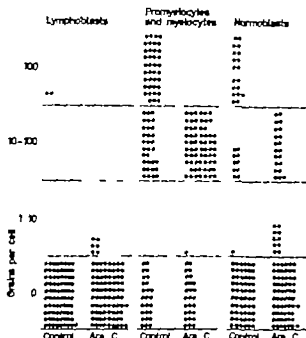


Fig 1 Distribution of grain counts in leukaemic lymphoblasts and normal haemic precursor cells from case 1. Comparison between cultures treated with cytosine arabinoside (Ara-C) and controls. ³H-thymidine labelling.

arabinoside was less than 1 %, compared to 4–26 % in the control cultures, and the few labelled cells seen in the treated cultures had a much lower intensity of labelling. The cultures from the remaining case of acute leukaemia (case 1) was of special interest since, as described above, the cultures contained apparently normal myeloid and erythroid precursors as well as the leukaemic lymphoblasts. Thus the effects of the drug on ³H thymidine uptake could be observed on different haemic cell types from the same individual and in the same culture. The results are illustrated in figure 1. In the control culture, 12% of the leukaemic lymphoblasts were heavily labelled (> 100 grains per cell) while 7% were lightly labelled (1–10 grains per cell) the remaining cells were unlabelled. (The grains in the lightly labelled cells could not have been due to background which was very low in this preparation.) The labelled cells thus did not form a continuous frequency distribution but consisted of two distinct groups. The lightly labelled cells therefore could not represent cells which were just



Fig. 2. Distribution of grain counts in normal haemic precursor cells from normal marrow. Comparison between cultures treated with cytosine arabinoside (Ara-C) and controls. ^{3}H-thymidine labelling

entering or leaving S phase during incubation with H-thymidine. The most probable explanation is that they were cells which have begun DNA synthesis but have been arrested in S phase. Arrest in S phase has been noted in leukaemic cells [6] although in normal cells once DNA synthesis is begun it will usually continue until the process is complete. The effect of treating the culture with cytosine arabinoside is completely to abolish the heavily labelled group in the leukaemic lymphoblasts while the lightly labelled group is slightly increased to 13%. Thus the effect on the leukaemic cells was a marked inhibition of DNA synthesis. In the same culture, DNA synthesis in myeloid and erythroid precursor cells appear only moderately inhibited (table III fig 1). In the experiment on normal marrow (fig 2) the effect of cytosine arabinoside in inhibiting ^{3}H thymidine uptake is slightly greater especially in normoblasts but the general pattern is essentially similar to that of the myeloid and erythroid precursors in case 1 i.e.

the inhibition is only partial whereas in the leukaemic cells it appeared to be virtually complete.

Discussion

CHU and FISCHER [7] studied the effects of cytosine arabinoside on L5178Y mouse leukaemia cells *in vitro* and reported depression of DNA synthesis in the presence of this drug. This action was reversed by the addition of deoxycytidine and they proposed that the drug acts by inhibiting the conversion of cytidylic acid to deoxycytidylic acid. Reversal of action by deoxycytidine was also observed *in vivo* [8]. This mechanism of action fits in well with the observation that RNA synthesis is not affected by this drug [7] although cytosine is present in both DNA and RNA. CLINE [9] however reported inhibition of RNA synthesis by cytosine arabinoside in human leukaemic cells and suggested that this might form the basis of an *in vitro* sensitivity test system. The incubation period used by him was however much longer than in the present experiment and it is probable that the inhibition of RNA synthesis reported by him is secondary to inhibition of DNA synthesis.

The chief interest in the present study lies in the finding of increased *in vitro* sensitivity to cytosine arabinoside of human leukaemic blast cells compared to normal myeloid and erythroid precursor cells, as judged by the degree of inhibition of DNA synthesis. The reason for this apparent selectivity is not clear. It has been suggested that the rate of growth of leukaemic cells may be related to availability of essential metabolites such as deoxycytidine [8]. Differences in pool kinetics of these metabolites might thus form a basis for selective inhibition of DNA synthesis by cytosine arabinoside. The observed occurrence of leukaemic cells arrested in S phase [6] might also indicate increased vulnerability of leukaemic cells during this part of the cell cycle compared to normal haemic precursor cells.

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Summary

The effect of cytosine arabinoside on nucleic acid synthesis in human leucocytes was studied using *in vitro* cultures of blast cells from 5 cases of acute leukaemia and normal haemic precursor cells. A difference in the degree of inhibition of DNA synthesis by cytosine

arabinoside in different cell types was observed, the inhibition being much more marked in the leukaemic cells compared with normal promyelocytes, myelocytes and normoblasts. RNA synthesis appears unaffected by the drug.

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On the PAS Reaction in Acute Paraerythroblastic Hemoblastoses

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Recently [1 2] we reported about enzyme cytochemical investigations on acute erythremias (di Guglielmo's disease) With ordinarily stained blood smears and autopsy sections these cases at first could not be definitely classified because the main cell type was an undifferentiated basophilic blast cell. As the naphthol AS-D chloroacetate esterase reaction revealed completely negative results, the cells obviously were not related to the neutrophilic cell strain. Applying the non-specific acid phosphatase reaction, however the blasts were strongly positive within a small paranuclear area (fig 1a) In normal bone marrow smears only proerythroblasts display a pattern of acid phosphatase reactivity comparable to that found in those blasts. Furthermore, infiltrates of parenchymatous organs were intermingled with maturing erythroblasts, which could be detected by their typical perinuclear α naphthyl-acetate esterase activity Therefore we considered the cases as extremely immature erythremias. Reinvestigating peripheral blood smears of one of our cases we found PAS-positive erythroblasts and erythrocytes (fig 1b) The reaction was diffuse in erythrocytes and normoblasts whereas basophilic macroblasts and a small number of undifferentiated blasts exhibited a coarse granular positivity Similar results on blood and bone marrow smears of 2 more of our cases have been attained by Drescher [3]

PAS-positive erythroblasts and erythrocytes are held by many authors to be quite diagnostic for erythremias and erythroleukemias [4 5, 6 7 8] Thus, the demonstration of PAS-positive material in blast cells, erythroblasts and erythrocytes of our cases together with the enzyme cytochemical findings mentioned above once more underline



Fig 1 Peripheral blood smear of extremely immature acute erythremia. Non-specific acid phosphatase reaction. Note the typical paranuclear positivity *b* Periodic acid-Schiff reaction. Two positive paraerythroblasts (left) large positive blast cell and positive erythrocyte (middle) and two positive normoblasts (right) (1 400)

our suggestion that these hemoblastoses can be well classified as immature erythremias In consequence the undifferentiated blasts exhibiting a strongly paranuclear acid phosphatase reaction indeed represent atypical erythroblasts as we proposed before [1 2]

Our report shows that certain cases of immature erythremias probably will be mistaken for acute undifferentiated granulocytic or lymphatic leukemias, if only common hematological stains are applied. In order to avoid such difficulties cytochemical techniques should be used as additional diagnostic tools. So there could certainly be achieved a much more proper classification of the various types of acute hemoblastoses than is possible by ordinary stains alone. We believe our cases of acute erythremias to be appropriate examples for the importance of cytochemistry in hematology

Summary

The value of cytochemical reactions for the diagnosis of acute, extremely immature erythremias is briefly discussed with special reference to the non-specific acid phosphatase reaction and to the PAS reaction. This is illustrated by representative case.

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Glucose-6-Phosphate Dehydrogenase Deficiency in Italy

A Study of the Distribution and Severity of the Enzymatic Defect

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G. Gaetani and G. Paravidino

Susceptibility to drug-induced haemolysis linked to a deficiency of glucose-6-phosphate dehydrogenase (G6PD) in red cells is inherited following a pattern which has aroused interest and new theories among geneticists [1] Males usually display full expression of the trait, while heterozygous females show a wide range of the metabolic abnormality A deficiency of G6PD cuts off the NADPH supply necessary for the protection of haemoglobin from oxidants in the presence of haemolytic drugs Heinz bodies are formed and the red cells are destroyed. It is known that if the trait has a slight degree of expression or if haemolytic drugs are never administered to G6PD-deficient subjects the metabolic defect of the red cells may never produce clinical effects. Thus, in order to evaluate the occurrence of drug sensitivity in a large population it is necessary to apply biochemical screening tests.

The defect is geographically widespread although limited to certain ethnic groups. American Negroes, Sephardic Jews, Chinese, Mediterranean populations (Egyptians, Sardinians, Greeks) have the highest incidence of the trait. In some areas of the Mediterranean (Sardinia) G6PD deficiency occurs also as favism. The incidence of G6PD deficiency in some groups of the Italian population (Sardinians, Ligurians, Tuscans, Umbrians) has been determined by Sansone et al [2] Contu et al. [3] Brunetti et al [4] and Siniscalco et al [5]

The main purpose of the present investigation is to study the susceptibility of Italians of different regional extraction to drug induced haemolysis. The considerable number of drug-sensitive Caucasians detected has provided a basis not only for the study of enzymes

of the red cells but also of leucocytes and platelets. Investigations on the clinical course of drug induced haemolytic crises in Sardinians [6] on the hepatic and splenic share in drug-induced haemolysis [7] and on blood group patterns of sensitive Sardinians [8] have been reported in other papers.

Methods

Sampling and Screening procedures. For this investigation advantage was taken both of the presence among the Genoese inhabitants of several groups of different regional extraction and of the possibility of direct connections with Sardinia and Sicily.

Blood collection was performed using specially prepared test tubes containing 1.5 ml of ACD-inosine solution, which were filled to the 10 ml mark with blood. When the blood collection was performed in Sardinia or in Sicily the samples were shipped by air in refrigerated container and reached our laboratory within 12 h of collection. Samples were stored at +4 C for not more than 3 days.

The methaemoglobin reduction test (MetHb test) according to Brewer *et al.* [9] as modified by us [10] was used. G6PD was measured on most of the subjects giving positive MetHb test for G6PD deficiency and on large number of negative subjects who were taken as controls. 6-Phosphogluconate dehydrogenase (6PGD) glutathione reductase (GSSG reductase) and methaemoglobin reductase (MetHb reductase) were determined on several cases.

The data reported here have been obtained by strictly random sampling. Relatives of both controls and G6PD-sensitive subjects have not been taken into account. Sometimes the relatives of sensitive subjects were also studied, but the data have been utilized for biochemical studies only.

Most of our blood samples came from the following sources 1. in and out patients of the Istituto Patologia Medica of the University Genoa 2. Transfusion Center AVIS, Genoa 3. Blood Bank of the Italian Red Cross, Genoa 4. medical students of the University of Genoa 5. patients from the Surgical Clinic and from the Obstetric wards of the University of Sassari (Sardinia) 6. patients from the Medical Clinic of the University of Cagliari (Sardinia) 7. patients from the Istituto Patologia Medica of the University of Catania (Sicily). We are indebted to the medical staff of these units for the collection of the blood specimens.

Sardinians formed a high percentage of our cases. This was intended in order to have large group for biochemical and clinical studies of drug-sensitive subjects.

Techniques. The modifications of the MetHb test, consisting in the use of vials containing lyophilized weighed amounts of sodium nitrite and methylene blue, have been described elsewhere [10]. Our normal values of remaining methaemoglobin ranged from 0 to 7% [11]. G6PD and 6PGD activities were determined according to KORNBERG and HORECKER [12]. GSSG and MetHb reductase activities were measured according to BONSIGNORE *et al.* [13]. Collection and enzymatic assays of leucocytes and platelets were performed according to MARKS and GROSS [14] and to WURZEL *et al.* [15].

Results

The MetHb test was carried out on 5 412 Italians. In 2 772 Italians of different regional extraction 14 subjects gave a positive

Table I Frequency distribution of G6PD deficiency between males and females in different geographical regions of Italy

Region	Screened males	MetHb test positive males	Percent	Screened females	MetHb test positive females	Percent	All cases	MetHb test positive cases	Percent
North Italy Piemont Lombardia, Liguria	625	2	0.32	376	4	1.06	1001	6	0,60
Emilia, Veneto	146	0	0	157	1	0.63	303	1	0.33
Central Italy Toscana, Lazio, Marche Umbria	112	1	0.89	94	0	0	206	1	0.48
South Italy Campania, Calabria, Abruzzi, Puglie, Basilicata	117	0	0	114	1	0.87	231	1	0.43
Continental Italians of mixed extraction	300	0	0	220	0	0	520	0	0
	1900	3	0.23	961	6	0.62	2261	9	0.39
Sicily	300	4	1.33	198	1	0.50	498	5	1.0
Sardinia Cagliari	481	120	24.91	232	64	27.58	713	184	25.80
Nuoro	124	8	6.40	83	12	14.45	207	20	9.66
Sassari	520	30	5.76	1034	112	10.83	1554	142	9.13
Sardinians of mixed extraction	44	9	20.40	122	24	19.67	166	33	19.87
	1169	167	14.28	1471	212	14.41	2610	379	14.33

result, with a frequency of 0 4‰. In 2 640 Sardinians the enzymatic defect was present in 379 subjects, with a frequency of 14.35 ‰. The frequency distribution between males and females in different regions is shown in table I and figure 1 Continental Italians show a low incidence of G6PD deficiency in contrast with the striking incidence found among Sardinians. A slightly higher frequency has been found in Sicily As far as Sicily is concerned the screening was mainly performed on subjects from the Catania province. In Sardinia the highest frequency is found in the province of Cagliari.

G6PD determinations were performed on 555 normal subjects and on 295 subjects with a positive MetHb test (table II) In males no

Table II. Quantitative assay of four red cell enzymes in normal and G6PD-deficient subjects

Subjects	Number of cases	G6PD (U/g Hb/min) mean ± SD	χ	
Normal	555	10.56 ± 2.47	h.s. (Normal vs Homozygous)	h.s. (Normal vs Heterozygous)
Homozygous	113	0.45 ± 0.39	h.s. (Homozygous vs Heterozygous)	
Heterozygous	182	5.61 ± 1.62		
		6-PGD (U/g Hb/min) mean ± SD		
Normal	117	6.57 ± 1.88	n.s. (Normal vs Homozygous)	n.s. (Normal vs Heterozygous)
Homozygous	51	7 16 ± 2.05	n.s. (Homozygous vs Heterozygous)	
Heterozygous	40	5.92 ± 1 40		
		GSSG reductase (U/g Hb/min) mean ± SD		
Normal	55	4.95 ± 1 79		
Homozygous	28	7 17 ± 2.24		
Heterozygous	8	5.21 ± 2.97		
		***MetHb reductase* (U/g Hb/min) mean ± SD**		
Normal	33	3.79 ± 1.22	n.s.	
Homozygous and heterozygous	16	2.93 ± 1.54		

h.s. highly significant significant n.s. not significant.

Table III G6PD and 6PGD levels in leukocytes and platelets of normal and G6PD-deficient subjects

Subjects	Number of cases	G6PD (U/10^6cells/min) mean ± SD	χ	
Leukocytes				
Normal	25	42.5 ± 12.2	h.s.	
Homozygous	11	6.3 ± 1.84	h.s.	h.s.
Heterozygous	4	14.5 ± 3.0		
		6PGD (U/10^6cells/min) mean ± SD		
Normal	23	16.5 ± 6.2	n.s.	
Homozygous	11	9.5 ± 3.64	n.s.	n.s.
Heterozygous	3	10.6 ± 3.78		
		G6PD (U/10^6platelets/min) mean		
Platelets				
Normal	5	0.475	h.s.	
Homozygous and heterozygous	7	0.106		
		6PGD (U/10^6platelets/min) mean		
Normal	3	0.081	n.s.	
Homozygous and heterozygous	4	0.060		

h.s. highly significant significant n.s. not significant.

subject with a positive MetHb test had G6PD activity values higher than 3 U/g Hb/min a few had values ranging from 1 to 3 U the greatest number showed values below 1 U/g Hb/min. In two males, red cell G6PD activity was higher than 5 U/g Hb/min. One of these cases was a probably Klinefelter syndrome. Table II also shows the results of the determinations of other red cell enzymes. No significant difference has been detected in the red cell 6PGD activity of 117 normal controls and 91 primaquine-sensitive subjects. NADPH-linked GSSG reductase was found significantly higher in the red cells of 28 G6PD-deficient males, as compared to the normal values of



Fig. 1. Distribution of screened cases (black dots) and percentage of G6PD deficiency in the Italian regions.

55 controls. The slight decrease of the mean values of NADPH-linked MetHb reductase in 16 G6PD-deficient males was not statistically significant, as compared with the activity found in the red cells of 33 controls.

In leukocytes and platelets of primaquine-sensitive subjects the G6PD activity was significantly lower compared to the activity of normal controls (table III). 6PGD activity in the leukocytes of 11 G6PD-deficient males and 3 heterozygous females yielded mean values which were not statistically lower compared to the normal controls.

Discussion

Among the Italian population Sardinians have a strikingly higher incidence (14.35 %) of G6PD deficiency than is found among continental Italians who are similar in this respect, to other Caucasian

populations [14 17] It is interesting to note the somewhat higher frequency (1%) of G6PD deficiency among Sicilians. This is comparable to the frequency found in other littoral mediterranean populations (Greeks, Algerians) [18, 19 20]

The distribution of the enzymatic defect among the three main provinces of Sardinia is uneven. SINISCALCO *et al.* [5] have demonstrated *that the incidence of the defect is quite variable from village to village.* However the large population sample and the proper randomization of our screening suggest that the frequency of the defect here reported is significant for the Sardinian population as a whole.

In Sardinia malaria was widespread until the end of the Second World War until then therefore both G6PD deficiency and malaria had a high incidence. In other Italian regions, e.g. Tuscany the low lands around Rome and the region in the heel of the boot, where malaria was common in the past, we have found a very low incidence of G6PD deficiency These findings however are not in contrast with the malaria hypothesis as stated by ALLISON [21] and MOTULSKY [17]

Data here presented outline the existence of considerable variations in the incidence of G6PD deficiency within a relatively small ethnic group settled in a limited geographic area. In the case of Italians suspected of G6PD deficiency therefore, the regional extraction should be carefully investigated.

From a practical point of view the high frequency of the enzymatic defect is a considerable disadvantage expecially for blood banks in affected areas. Transfusion of G6PD-deficient red cells constitutes a hazard for patients receiving drugs which rapidly destroy such red cells [22]

In heterozygous Sardinian females the degree of red cell G6PD deficiency was comparable to that found in intermediate Negro females (i.e. 53% of the enzymatic activity of normal controls) Among heterozygous Sardinian females, we were able to find some cases with a G6PD activity which overlapped normal values [16]

The G6PD deficiency of the red cells of Sardinian males was found to be very marked. Values not higher than 3 U were found in very few instances, but in the majority of the deficient males no G6PD activity could be detected in the red cells[1] The degree of the enzymatic defect

[1] These data suggest that the mild type of G6PD deficiency although observed for the first time in Italy [29] and commonly observed in the Greek population [19], does not occur frequently in the Sardinians.

in the red cells of Sardinian males explains the severity of the haemolytic crisis induced by primaquine in contrast to the self limited character of the drug-induced disorder in Negroes. In a mutant Sardinian male, even young red cells were susceptible to haemolysis. In such individuals extremely severe haemolytic crises may occur recalling the life threatening haemolytic episodes sometimes seen in favism [6] This greater severity of the enzymatic defect in Caucasians and its linkage to severe haemolytic episodes is confirmed by BURKA *et al.* [23] who noted among Caucasians a higher frequency of haemolytic crises than should be expected from the frequency of the trait. Transfusion experiments with ^{51}Cr tagged red cells collected immediately after the haemolytic crises, confirm the susceptibility of the younger red cells to drug induced haemolysis [6] The data on other enzymes of the red cells in Sardinians confirm the observations by SCHRIER *et al.* [28] *on the increased GSSG reductase activity of the deficient* red cells in Negroes, which may be possibly due to a compensating mechanism in the affected cells. The enzymatic defect involves not only the circulating red cells but also leukocytes, platelets, jejunal [24] and gastric mucosa [25] liver [14 25] spleen ad bonex marrow cells [26] (granuloblasts and erythroblasts) however in these subjects the red cell regeneration is not at all hampered In fact, in three G6PD-deficient Sardinian subjects, the calculated daily red cell production after drug induced haemolytic crises was between 2.8 and 3 1 times the normal red cell production [27]

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Summary

Susceptibility to drug-induced haemolysis was studied among 5 412 Italians. The highest incidence (14.35%) of G6PD deficiency was found, as expected, among Sardinians. Continental Italians hav ery low frequency (0.4%) of the defect, whereas Sicilians show somewhat higher incidence (1.0%) The degree of G6PD deficiency in the red cells of all *affected Caucasian males is extremely severe and is* probably correlated with the severity of the haemolytic episodes observed. The mild type of G6PD deficiency observed in the Greeks does not occurr in the Italians. Data here presented outline the existence of considerable variations in the incidence of G6PD deficiency within relatively small ethnic group like the Italians.

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Vitamin B_{12} Absorption Test[1]

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Before 1953, the only definite laboratory test for diagnosis of pernicious anemia (PA) was in vivo testing of patient's gastric juice for intrinsic factor (IF) activity, which was a cumbersome technique. Since the introduction of cobalt-labelled vitamin B_{12} in 1952 [8] four methods of evaluation of vitamin B_{12} absorption have been described: fecal excretion [8], urinary excretion [12], hepatic uptake [7] and plasma level determination of radioactive vitamin B_{12} [2, 5]. For obvious reasons the urinary excretion method of Schilling [12] (or its modifications) has been widely used. Disadvantages of this method are a) difficulties of collecting the 24 h urine specimen, b) low results in chronic kidney disease, c) administration of a large dose of cold vitamin B_{12} which alters the clinical and hematologic pictures. Plasma level determination after oral administration of labelled vitamin B_{12} does not have these disadvantages and is a direct measurement of vitamin B_{12} absorption.

In the past this test has had lack of accuracy because of low specific activity of the labelled vitamin but this had been obviated since the use of ^{57}Co. This is the preferable isotope when measurements are made by a spectrometric method, because of the single high peak energy spectrum at 0.123 Mev and low radiation dosage to organs [3, 4]. The purpose of this work is to obtain an accurate and practical ^{57}Co vitamin B_{12} plasma level test for clinical use.

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Materials and Methods

^{57}Co vitamin B_{12} had a specific activity of 12.7 to 20.1 µc/µg[1]. Each patient received 0.12 to 0.15 µg of labelled vitamin B_{12} (2.27–3.19 µc) diluted in 100 to 200 ml of water. No flushing dose of 'cold' vitamin B_{12} was given. Subjects did not receive any vitamin B_{12} injection during and 24 h prior to the test. Standard solutions were prepared by adding 0.1 ml of stock radioactive B_{12} to 100 ml of water.

Overnight fasting subjects were given ^{57}Co-vitamin B_{12} orally in the morning, following withdrawal of blood samples for serum folate, vitamin B_{12}, iron, Hb, Hct, BUN and oxalated sample for background plasma radioactivity. Breakfast was given 1 to 1½ h later. Ten ml of oxalated samples of blood were obtained every 2 hours for 12 h, then at 24 and 48 h. Four ml of standard solution and plasma samples were counted for 5 min using well-type gamma spectrometer. Background also was counted for 5 min and subtracted from each count. Total blood volume was calculated according to weight, height and sex of subject [10]. Plasma volume was calculated from predicted total blood volume and hematocrit. Percentage of radioactivity of given dose was calculated for total plasma volume. When plasma ^{57}Co-vitamin B_{12} level was low the test was repeated with addition of 10 mg intrinsic factor concentrate[3] to labelled vitamin B_{12} solution.

Three groups of subjects were studied.

1. Control group (25 cases) consisted of 3 healthy employees and 22 patients admitted to the hospital for a variety of medical and surgical diseases, who were in relatively good health. None had evidence of malabsorption. There were 22 males and 3 females between the ages of 15 and 78 years. When the absorption test was done, Hb ranged from 10.4 to 15.2 g% and only one of 25 patients had elevated BUN (49 mg%).

2. Pernicious anemia group (10 cases) consisted of 9 females and one male between the ages of 49 and 85 years. Patients had been treated with vitamin B_{12} from 2 days to 14 years. Initial Hb was from 3.2 to 7.3 g%. All had megaloblastic bone marrow, macrocytic anemia and histamine refractory achlorhydria. They responded to vitamin B_{12} therapy with reticulocytosis and elevation of Hb, and were subsequently maintained with vitamin B_{12} therapy. When the absorption test was performed Hb ranged between 7.1 to 15.7 g%. Four patients had elevated BUN (up to 50 mg%). Results of Schilling test were unreliable in most cases, even on repeated instances, because of poor cooperation of patients. Initial serum vitamin B_{12}, folic acid, LDH and iron determinations were performed in only 5 patients. Serum vitamin B_{12} levels ranged between 10 and 50 pg/ml, LDH was elevated in all, and serum folic acid was 21 ng/ml or higher in 4 and low in one (3.5 ng/ml). Four patients had normal or slightly elevated serum iron levels and one patient had low value. Anti-IF and anti-thyroid antibody determinations were performed on 7 patients and were positive in 3 and 2 subjects, respectively.

3. Miscellaneous group (7 cases): four patients with subtotal gastrectomy performed 8 to 20 years previously for complicated peptic ulcer. There were 3 males and one female between the ages of 55 and 75 years. When the test was performed Hb was above 12.5 g% in two and 7.0 g% in remaining two. Serum vitamin B_{12} values were below 100 pg/ml in two cases, slightly reduced in one and normal in the fourth person. Two of 4 subjects had been diagnosed as megaloblastic anemia in the past and treated with vitamin B_{12}. Two patients with malabsorption syndrome were studied: one was 32-year-old male with chronic alcoholism and diffuse calcification of the pancreas; the second patient was a 74-year-old female

Radioactive vitamin B_{12} received from Merck and Co., Inc., Rahway, N.J. and Abbott Laboratories, Chicago, Illinois.

Received from Dr. Leon Ellenbogen, Lederle Laboratories, Pearl River, N.Y. (active at 5.0 mg level).

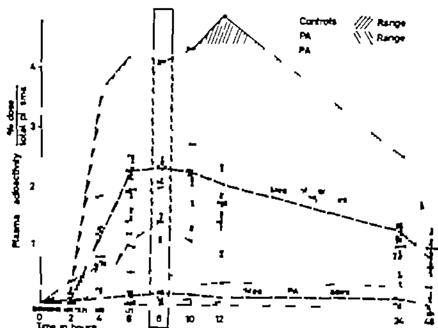


Fig 1 Results of [57]Co-vitamin B_{12} absorption-plasma level test in 25 control subjects and 10 patients with PA without and with IF Plasma radioactivity is calculated as % of oral dose in total plasma. Best separation of the two groups is obtained at 8 h following the oral dose. (Numbers in parentheses indicate number of cases with zero absorption represented with single figure).

treated for anemia for 20 years with vitamin B_{12} injections. The latter patient never had gastric analysis, bone marrow examination or tests for malabsorption. Both subjects had normal Hb and serum vitamin B_{12} levels at time of test. Last patient was 47-year-old female with nutritional megaloblastic anemia, admitted with Hb 2.9 g% and Hct of 14%, serum folic acid 1 1 ng/ml and serum B_{12} 45 pg/ml, typical megaloblastic-macrocytic morphology elevated LDH, free acid in gastric juice and spinal cord disease. She was transfused with packed red cells and 1,000 μg of cyanocobalamin were given intramuscularly for 8 days without any response. Excellent reticulocytosis was obtained with folic acid therapy The latter rose to 20% with subsequent increase of hemoglobin, improvement of spinal cord disease and complete normalization of bone marrow and peripheral blood.

Results

1 In the *control group* of 25 cases the highest plasma levels were reached at 8 h with a mean of 2.33% and a range of 1 41–4 12% (fig 1) After 4 h there was no overlapping between control and perni

Table I Comparison of results of recent studies on

	Dose	Flushing dose B_{12} (μg) – time (h)	Ref. h	Amount of plasma counted ml
Ostergaard-Kristensen [11]	0.5 μc/0.5 μg	1000–10	12	4
Armstrong & Woodliff [1]	1.0 μc/1.0 μg	1000–6	8	5
Coopland [3]	1.0 μc/1.0 μg	1000–6	8	5
Workman & Rubin [14]	1.0 μc/1.0 μg	–	8	4
Woodliff & Armstrong [15]	1.0 μc/1.0 μg	1000–6	8	5
McCurdy [9]	0.5 μc/0.5 μg	1000–1	8–12	4
Forshow & Harwood [6]	0.5 μc/0.5 μg	1000–2	8	5
Present study	2.27–3.19 μc 0.12–0.25 μg	–	8	4

cious anemia groups, and the best separation of these two groups was seen at 8 h. With few exceptions the 8 hour specimen gave highest value in all groups.

2 In the group of *pernicious anemia* patients the mean plasma level at 8 h was 0.19% with a range of 0.00–0.60% The highest value in this group was well below the lowest level of normal subjects. When IF and ^{57}Co-vitamin B_{12} were administered to patients with PA, the mean level in plasma at 8 h rose to 2.28% with a range of 1 11–4 44%. Eight were within normal limits and two remained at subnormal levels.

3 *Miscellaneous group* (7 cases) Two of 4 patients with sub-total gastrectomy showed normal vitamin B_{12} absorption (1 75 and 1.57% at 8 h) The third person had low absorption (0.36% at 8 h) which was normalized with IF (1 47%) The fourth case showed no absorption with or without IF Two patients with malabsorption syndrome showed very low plasma radioactivity at 8 h with and without IF (0.10% and lower) One patient with nutritional megaloblastic anemia showed normal absorption (3.53% at 8 h)

*Cobalt [illegible] B_{12} absorption-plasma level test

Calculation	PA		PA with IF		Normal controls	
	Range	Mean	Range	Mean	Range	Mean
1000 ml of plasma	0.00–0.33	0.1	0.9[illegible]6	1	1.01[illegible]38	1.3
1000 ml of plasma	0.03–0.17	0.10	0[illegible]3–1	1	0.73–1[illegible]	1[illegible]
1000 ml of plasma	0.0[illegible]–0.4	0.17	0.53–[illegible]00	1[illegible]	0.[illegible]19	1.25
[illegible]1000 ml of plasma	0.00–0.19	0.16	0.43–[illegible]1	[illegible]5	0.55–0.93	0.75
1000 ml of plasma	0.00–0.40	0.14			0.73–[illegible]30	1.1
cpm	1[illegible]1	4	[illegible]3–8	43	15–130	51
cpm	0–19		4–4		25–64	
[illegible]total plasma	0.00–0.60	0.19	1.11–4.44	[illegible]25	1.41–[illegible]1	[illegible]33

Discussion

The purpose of vitamin B_{12} absorption tests is to diagnose and classify vitamin B_{12} deficiency in 3 main groups by 3 characteristic absorption patterns

1 Normal pattern which indicates the presence of normal amount of IF secretion and normal absorptive function in gastrointestinal tract for vitamin B_{12} this is seen in normal subjects, in vegetarians and persons with nutritional megaloblastic anemia.

2 PA pattern Low absorption without IF and normalization of absorption when the test is repeated with IF This indicates deficient IF secretion and normal absorptive function of gastrointestinal tract. This is typically seen in PA and also following total gastrectomy

3 Malabsorption pattern Low absorption with and without IF which indicates defective function of gastro-intestinal tract with [illegible]

or deficient IF secretion. This is seen in different types of malabsorption syndromes, including familial selective malabsorption of vitamin B_{12}. Coexistence of PA and malabsorption cannot be diagnosed by any kind of vitamin B_{12} absorption test. The only way to diagnose this combination is by demonstration of deficiency of IF in the gastric juice of the patient by *in vitro* or *in vivo* IF determination.

In our study ^{57}Co-vitamin B_{12} absorption plasma level test on 42 subjects gave 3 distinctive absorption patterns with clear-cut separation of each group and no overlapping at 8 h. With this limited number of cases no equivocal results were obtained. Normalization of absorption was observed in 8 cases of PA when the test was repeated with IF and in remaining 2 patients the values were subnormal but still distinctly higher than the original low values. Recent studies of different workers on ^{57}Co-vitamin B_{12} absorption plasma level tests gave similar results but with lower plasma radioactivity values and in some cases equivocal results [1, 3, 6, 9, 11, 13, 14]. Probably this is due to use of vitamin B_{12} with lower specific activity and lower dosage (0.5 μc/0.5 μg to 1.0 μc/1.0 μg) and differences in methods of calculation of absorbed vitamin B_{12}. This has generally been done per 1000 ml of plasma [1, 11, 13, 14] or expressed as cpm in 4 or 5 ml of plasma [6, 9]. A flushing dose of cold vitamin B_{12} injection was frequently administered at different time intervals and occasionally carbocal was also given before the test [1, 3]. Generally the 8 hour plasma sample was used as reference for the best separation of normal group from PA.

In our study ^{57}Co-vitamin B_{12} with a higher specific activity was used which contained a physiologic amount of vitamin B_{12}. No flushing dose of cold vitamin B_{12} or carbocal was given. Thus the experimental conditions were more nearly physiologic. Plasma level was calculated per total plasma volume which better reflects absorbed vitamin B_{12}. Although calculation of total blood volume was made from a table of weight, height and sex of subjects which has some inherent error, results gave a better separation between control and PA groups. Comparison of results of other workers and ours is shown in table I. No significant difference of vitamin B_{12} absorption was observed between subjects with normal and high BUN. There was no relation between vitamin B_{12} absorption and serum B_{12} or folate level at the time the test was performed. In one patient with subtotal gastrectomy vitamin B_{12} absorption was not normalized when the test was repeated with IF. This is probably due to associated malabsorption in the ileum.

Summary

A modified ^{57}Co-vitamin B_{12} absorption plasma level test was described in three groups of subjects. Eight hours following the oral dose there was a good separation of the control group from PA patients without overlapping. The former showed an absorption range of 1.41–4.12%, with a mean of 2.33%. PA group had a range of 0.00–0.60%, with a mean of 0.19%. When the test was repeated with IF in this latter group, a range of 1.11–4.44% with a mean of 2.28% was obtained, an increase of 3.8 fold or more in each case. Our findings and results of other studies were compared and discussed. The test can routinely be used with only two blood samples taken at zero and 8 h.

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Addendum

Since preparation of this manuscript, the test has routinely been used in this institution. The results of both old and new groups combined were as follows (6b) Mean of 48 control subjects was 2.45% with range of 1.39–4.12%. Mean and range of 27 PA patients without IF were 0.16% and 0.00–0.60% respectively When the test was repeated with IF mean of 2.02% with range of 1.11–4.44% was obtained (with 5 subnormal values) Test completed on 11 new patients in miscellaneous group gave similar results to that of 7 cases previously described, except one patient with hypothyroidism who had subnormal absorption (1.14%) without IF

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Functional and Cellular Alterations Produced by Phytohemagglutinin

III. Hematologic Findings in Mice Receiving PHA

B. B. Lozzio, E. A. Machado and A. L. Chernoff

*The treatment of the bone marrow depression with phytohemagglu*tinin (PHA) in human [4–6] and animals [7, 15, 20] has been attempted with variable results. It appears that PHA may produce improve*ment in drug induced marrow failure, but it is not likely to do so in* idiopathic aplastic anemia or sublethally irradiated animals.

Very little is known about the effects of PHA on normal hematopoiesis. An increase in the mitotic rate of marrow cells has been found in normal young rats after PHA administration [2]. The injection of PHA may cause leucopenia and anemia in rodents [3, 8–9].

This communication describes the alteration of the hematopoiesis of the mouse resulting from the administration of PHA.

Materials and Methods

Animals and mitogens. The animals used in these experiments were 4-month-old male mice of the C3H/HeJax inbred strain. Bacto-phytohemagglutinin (Difco Lab., Detroit, Mich.) *in the M and P forms was given to experimental mice in a single dose of 30 mg/* 100 g body weight. Control animals were injected with 1.5 ml sterile saline per 100 g body weight, or inactivated mitogen (30 mg/100 g). Inactivation of the hemagglutinating and mitogenic activities of PHA was accomplished by boiling PHA solution (20 mg/ml) at 100°C for 30 min [13]. In all experiments mitogens and saline were given to mice i.p.

Hematologic studies. Hematologic studies were carried out in controls and mice treated *with PHA-P and PHA-M. The same mice were studied at each time interval. Baseline*

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hematologic values were determined twice prior to treatment. Subsequent blood samples were drawn at 24 h and at 3 day intervals for 12 days. Hemoglobin and hematocrit values, differential counts, and reticulocyte number were determined by standard techniques. Erythrocytes and leucocytes were enumerated in Coulter counter The survival time of homologous erythrocytes was studied in both control and PHA-treated animals. Homologous erythrocytes were obtained from normal mice and labeling was performed by adding 10 µCi of ^{51}Cr/ml of blood (specific activity 200 mCi/mg Cr) in ACD solution as described previously [10 11] Recipient animals were injected with 0.2 ml of 50 percent suspension of erythrocytes in saline. The first blood sample (100% value) was taken 20 h later following which PHA M, PHA-P saline, or inactivated PHA was injected. Subsequent blood samples (20 µl each) were taken t 3 day intervals for 15 days. At the end of that period the animals were sacrificed by total exsanguination. The liver spleen, lungs, kidneys, and whole skeleton were removed and digested in nitric acid as indicated previously [10] Blood samples and aliquots of the organs were counted for 5 min in an automatic γ counting system. Total organ radioactivity was determined by multiplying the activity of tissue samples by the total amount of nitric acid used. The sequestration of ^{51}Cr was expressed as percentage of the total radioactivity injected.

Microscopic examination. Groups of 5 mice were killed at 24 h and at 3 day intervals for 15 days following PHA-P PHA M, inactivated PHA or saline administration. The spleen and the femur were fixed in 10% phosphate buffer formalin (pH 7) solution, embedded in paraffin and stained with hematoxylin-eosin.

Statistic. The data are presented as mean ± one standard error

Results

Hematologic observations The mean half life (T ½) of homologous erythrocytes in 15 control mice was 13 9±0 8 days. Five mice treated with PHA M had erythrocyte survival times similar to those of controls (T ½=13 8±0 6 days) The administration of PHA P produced a significant ($p<0\ 001$) shortening of the erythrocyte survival time (T ½=10 0±0.5 days) in the 10 mice studied The decrease of the erythrocyte survival time was associated with an enhanced deposition of ^{51}Cr in the liver (10 6±0 4%) and spleen (22 9±1 4%) The uptake of ^{51}Cr by the liver (6 7±0 2%) spleen (13 7±1 2%) and skeleton (9 8±0 6%) was nearly identical in controls and mice receiving PHA M Lungs and kidneys accounted for 0 70 to 1 47% of the total radioactivity injected in all mice studied. The hematocrit values (42 8±0 8%) hemoglobin concentration (14 6±0 4 g %) and number of erythrocytes (7 30±0 08×10^6/mm^3) of mice receiving PHA M were similar to those found in the control groups. Mice given PHA P showed significantly ($p<0\ 001$) lower hematocrits (37 6±0.5%) hemoglobin values (11 4±0 2 g%) and erythrocyte counts (5.57±0 07×10^6/mm^3) during the 9 to 12-day period Similar reticulocyte

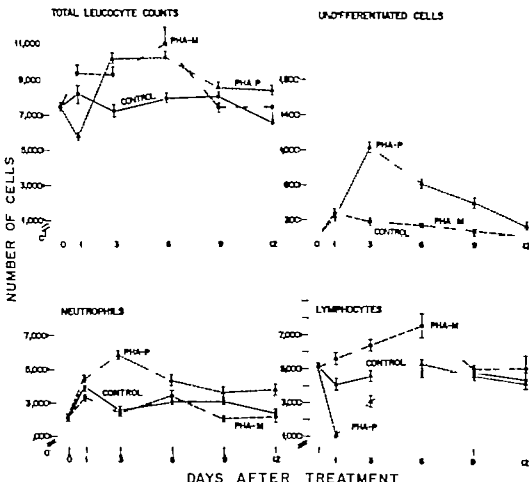


Fig 1 Leucocyte changes after PHA administration. Total leucocyte counts ('O' time) is the mean ± SE of 145 mice. Neutrophils, lymphocytes and undifferentiated cell values of controls ('O' time) is the mean ± SE of 30 mice. Other points represent the mean ± SE obtained from 10 animals.

counts (4 16±0.25 to 6.09±0.37%) were observed in the groups of animals studied.

A single injection of saline or heat inactivated PHA produced mild leucocytosis in the 24 hour period. Differential counts showed slight neutrophilia and lymphopenia (fig 1) In 30 control animals small



Fig 2. Peripheral blood leucocytes from control mice. Normal neutrophils (a–b), lymphocytes (–e) and monocytes (f) (Wright stain, × 1 000)

lymphocytes were predominant and undifferentiated cells represented 0 14 % of the leucocyte population. Controls and PHA treated mice had similar numbers of monocytes (2–5%) eosinophils (0–1%) and basophils (0–0.2%)

The injection of PHA M produced leucocytosis at 24 h which reached a peak 6 days later with normalization on the 9th day Differential counts showed neutrophilia and marked lymphocytosis (fig 1) There was an increase of medium sized lymphocytes with a corresponding decrease in the percentage of small lymphocytes during the 1 to 9 day period of observation Undifferentiated cells and a few plasmacytoid lymphocytes appeared on the 3rd day in 8 of 10 mice examined.

The administration of PHA P produced leucopenia within 24 h, followed by marked leucocytosis by the 3rd and 6th days. The leucocytosis diminished in subsequent period of observation *Differential* counts indicated a striking neutrophilia by the 1st and 3rd days. The neutrophilia was accompanied by a marked lymphopenia (fig 1) An increase of the number of medium and large lymphocytes was observed in the 6 to 9 days period after only one injection of PHA P Similar number of small and medium lymphocytes was found beyond the 9th day At that time, an abnormal number of neutrophils persisted in the circulation. Numerous undifferentiated *cells* and *plasmacytoid* lymphocytes were seen in the 10 mice studied particularly in the 3 to 6 days period after PHA P administration They had largely disappeared from the blood stream by the 12th to 15th day (fig 1)

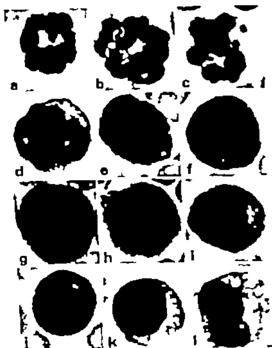


Fig. 3. Peripheral blood leucocytes from mice injected with PHA 1-6 days previously. Enlarged and hyperlobulated neutrophils (a–c). Undifferentiated cell (d–h). Most of the undifferentiated cells show delicate chromatin strands and deeply basophilic vacuolated cytoplasm. Plasmacytoid cells (i–k). Binucleated cell showing extensive cytoplasmic vacuolization (l) (Wright stain, 1,000)

The morphology of leucocytes of control mice is shown in figure 2 a–f. The injection of PHA P produced more marked cellular changes and affected a greater number of cells than did PHA M administration. One day after PHA administration the majority of the neutrophils appeared to be enlarged with hypersegmented nuclei (fig. 3 a–c). Some neutrophils showed cytoplasmic inclusions and vacuoles. These alterations were still present in several neutrophils on the 6th day.

The large undifferentiated cells rarely seen in control mice but not uncommon after PHA administration (fig. 3 d–h) had intensely basophilic, finely vacuolated cytoplasm and leptochromatic nuclei. Nucleoli were visible in a few of the cells. Some of the binucleat undifferentiat-ed cells showed extensive cytoplasmic vacuolization (fig. 3 l). Several

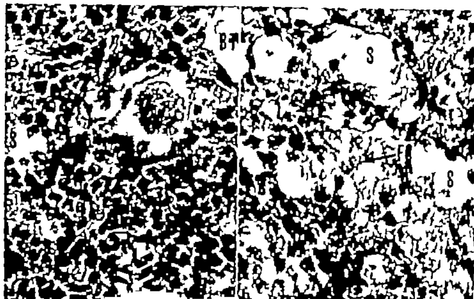


Fig 4 a Normal bone marrow with megakaryocyte (MG) myelopoietic tissue (MT) sinuses (S) and bone trabecule (BT) *b* Large foam cells (FC) in the bone marrow of mice injected with PHA 3 days previously (HE, ×400).

intermediate cells between normal lymphocytes and undifferentiated cells were also seen Numerous plasmacytoid lymphocytes (fig 3 i–k) were seen in PHA P treated animals. Plasmacytoid cells were not seen in control mice.

Bone marrow examination. Three days after the injection of PHA, large mononuclear cells with abundant foamy cytoplasm appeared in the bone marrow The accumulation of these cells gave the bone marrow of mice treated with PHA a clear vacuolated appearance (fig 4b) when compared with bone marrow from control animals (fig 4a) The foam cells gradually disappeared in subsequent periods of observation. The morphology of these cells makes them difficult to classify although they resembled macrophages. A slight decrease of myelopoietic cells was observed in 50% of the animals. Heat inactivated PHA produced no alterations of bone marrow cells

Splenic hematopoiesis By the 3rd day the splenic hematopoietic foci of mice given PHA were more numerous and larger than those observed in the spleens of mice treated with inactivated PHA or saline A marked rise of the cell number characterized the myelopoietic foci

of mice treated with PHA. A slight but constant increase of normoblasts was also observed 3 days after PHA. The stimulation of splenic hematopoiesis was noted to last as long as 12 days after a single injection of PHA.

Discussion

This study demonstrates that both PHA-M and PHA P administration produces striking hematopoietic alterations. However the changes observed are more marked with PHA P than with PHA M. PHA P contains stronger erythroagglutinating leucoagglutinating and mitogenic activities than PHA M which appears to be responsible for the hematologic modification observed.

The decrease of the erythrocyte survival time and the low RBC values found in the 9 to 12-day period after PHA P administration could be produced by erythroagglutination This suggestion is supported by the observation that the splenic red pulp shows an increase in red cell accumulation [12] and in Cr deposition in mice treated with PHA P However other mechanisms, such as the cytotoxic action of autologous lymphocytes stimulated by PHA, could also be involved in the decrease in erythrocyte levels. It has been shown that fowl erythrocytes are lysed when exposed to autologous lymphocytes in the presence of PHA provided they have been stimulated by PHA but not if PHA is omitted [16]

A transitory leucopenia was observed soon after the injection of PHA P Differential counts demonstrated lymphopenia and neutrophilia. These observations suggest that lymphocytes were removed from the circulation with a simultaneous release of neutrophils from depots. The transient leucopenia was followed by marked leucocytosis which appears to be largely due to the gradual increase in the number of neutrophils, lymphocytes and the appearance of undifferentiated cells. The leucocytosis coincided for a time with the stimulation of splenic hematopoiesis. During this period, the splenic white pulp was strikingly hyperplastic and large undifferentiated cells populated the lymphopoietic system [13] It is likely therefore, that a single large dose of PHA P results in the sequestration of erythrocytes and lymphocytes followed by stimulation of splenic myelopoiesis and lymphopoiesis. The splenic erythropoiesis also seems to be stimulated as indicated by an increased number of normoblasts

In contrast, stimulation of the bone marrow cells was not seen under these experimental conditions. The bone marrow became infiltrated with macrophage like cells (foam cells) which may represent intermediate or final stages of marrow cells stimulated by PHA. Indeed, the *in vitro* culture of rat bone marrow cells with PHA yields a much higher proportion of macrophages than when other tissues are cultured [14] hence the stem cell has been implicated among the groups of cells which respond to PHA by macrophage transformation Whether the macrophage like cells developing in the marrow of mice stimulated by PHA arise from lymphocytes or from other precursor cells is not known The foam cells differ from undifferentiated cells observed in the lymphopoietic system under the influence of PHA [13] and do not resemble any other type of bone marrow cells.

The spectrum of cells observed in the peripheral blood of mice given PHA was not observed in control animals. The abnormal cells, therefore appear to result from an *in vivo* effect of PHA. Whether the plasmacytoid cells are descendants of undifferentiated cells or are the result of a special *in vivo* blastogenesis of lymphocytes by PHA is not known. Similar cells have been observed in humans [1] and animals [11] after systemic exposure to another phytomitogen (pokeweed)

The majority of the neutrophils appear enlarged and hyperlobulated These alterations of the neutrophil may be caused by a toxic effect of the mitogen In this regard the addition of PHA to leucocyte cultures causes the neutrophils to degenerate while the lymphocytes undergo blastogenesis [19] PHA-stimulated lymphocytes are capable of increased histone acetylation and RNA synthesis [17–18] In contrast granulocytes (equine) exposed to PHA show a decrease in the rate of histoned acetylation and RNA synthesis [18] Such metabolic inhibitions may also be produced *in vivo* by PHA thus causing the morphologic changes observed

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Summary

A single intraperitoneal injection of phytohemagglutinin (PHA) in its M or P forms stimulated the normal splenic hematopoiesis of the mouse. *Bone marrow cells* evolved to

macrophage-like cells and no sign of erythropoietic stimulation was seen. An initial decrease of the number of leucocytes, followed by marked leucocytosis, was observed in mice given PHA-P. Differential counts indicated decrease of the absolute number of lymphocytes and rise of neutrophils. Neutrophilia and lymphocytosis followed the administration of PHA-M. The neutrophils showed enlargement and hypersegmentation. Numerous immature and plasmacytoid cells appeared in the peripheral blood. The injection of PHA-P caused anemia and shortening of the erythrocyte survival time. At the same dose level, PHA-P produced more marked hematologic alterations than did PHA-M.

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Hereditary Deficiency of Peroxidase and Phospholipids in Eosinophilic Granulocytes

B. Presentey and Lea Szapiro

The occurrence of a new anomaly of eosinophilic granulocytes consisting of nuclear hypersegmentation hypogranulation and negative peroxidase and phospholipid staining has been reported recently [4 6] A detailed cytochemical study of the anomaly has also been published [5]

The case to be described now shows a negative peroxidase and phospholipid staining of eosinophilic granules, the cyto-chemical description of which has been included in the abovementioned report [5]

Peroxidase tests are not part of the routine of our laboratory but since the discovery of the first anomaly we perform them periodically In this manner the present case was found. The frequency of these anomalies is not known because cytochemical and cytoenzymatic tests are not routinely performed in clinical laboratories. Only by performing these tests on a wide scale may the frequency be ascertained and further interesting cases discovered.

Case Report

An Israeli-born 2-year-old girl, daughter of young and healthy parents of Yemenite origin, complaining of lack of appetite for about fortnight, was examined in the children clinic. The family history shows no special complaints. The girl was born naturally after normal full term pregnancy and weighed 3,200 g birth. Up to the recent examination she suffered only mild complaints of the respiratory and digestive systems. On examination, the girl was found in general good health, was well nourished and her psycho-motor development was normal for her age. Apart from slight skin pallor the examination brought nothing to light.

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Laboratory examinations. Haemoglobin 9.9 g%, erythrocytes 4,500,000/mm³ leukocytes 12,800 mm³ (without shift to the left) eosinophiles 3%. Slight microcytosis and few poikilocytes. Eosinophiles gave negative results when tested for peroxidase by the methods of GRAHAM-KNOLL [1] and OSOGOO [3] and phospholipids by the method of LISON [2] (fig. 1, 2, 3) according to PRESSMAN [4, 5, 6]. BSR, urine tests, liver and kidney tests showed no pathological disturbances and stools were free from parasites.

The patient was treated with iron and folic acid she regained her appetite, the skin pallor disappeared and her haemoglobin count rose to 11 g%. Phospholipids and peroxidase staining of eosinophiles remained negative on repeated examinations.

Family study. The family tree is illustrated in figure 4. The proposita is in the fourth generation (at right). In all, 62 members belonging to 4 generations were examined. Cross-examination could not substantiate any suspicion for intermarriage between relatives. No morphological changes were observed in any of the eosinophiles. Absence of peroxidase and phospholipids was found in 2 of the mother's brothers. A brother and sister of the father also carry the defect.

Discussion

The genetic follow-up of a previously described family carrying the anomaly in its full morphological cytochemical manifestation [5] already inferred the autosomal recessive mode of inheritance. The evidence obtained in the present study agrees with this assumption.

As can be seen from the family tree, the anomaly of the eosinophiles has travelled from both the maternal and the paternal side of the proposita and makes its appearance only in the third generation two of the propositus uncles from the maternal side carried the anomaly although their children were normal. An uncle and an aunt of the propositus from the paternal side showed also the anomaly Both were not married. It is noteworthy that both parents of the propositus were also negative with respect to the eosinophilic anomaly The proposita herself was the only positive member in the fourth generation. These findings strongly suggest a recessive autosomal mode of inheritance The affected members being presumably homozygous and the phenotypically normal grandparents heterozygous. Such a mechanism could account also for the statistical aggregation of the anomaly (2 homozygous combinations out of 9–10 children, i.e. approximately 25%)

Fig. 1. Peroxidase staining by GRAHAM-KNOLL method.

Fig. 2. Peroxidase staining by OSOGOO method.

Fig. 3. Phospholipid staining with Sudan-Black B by LISON method.
a) Anomaly b) normal blood.
Only figure 1b is of peripheral blood smear the other figures are of buffy coats. The eosinophils of the anomaly are negative for peroxidase and phospholipids.

Fig. 4

Summary

A family with hereditary deficiency of peroxidase and phospholipids of the eosinophils is described. *The defect is not accompanied by any morphological anomalies. Its mode of* inheritance is autosomal and recessive. No connection between this defect and any form of illness has been found. All cases of this anomaly discovered to date were of Yemenite origin.

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Libri

A. Hittmair: Die Physiologie und Pathologie der Milz. Urban & Schwarzenberg, München, 1969. VII + 504 p., 7 fig. Preis DM 93.

Der Autor, der sich seit vielen Jahren als Hämatologe mit der Milz befasst, legt hier als Sonderdruck aus dem Handbuch der gesamten Hämatologie eine monographische Bearbeitung vor. Nach Darstellung von Morphologie und Physiologie der Milz werden besondere Funktionskreise des Organs, nämlich Zirkulation, Regulation und Abwehr herausgestellt. Unter pathologischen Bedingungen kann es zur Funktionsminderung, zur Überfunktion (Hypersplenismus) und Fehlfunktion (Dysplenismus) kommen. Schliesslich werden spezielle Milzpathologie und Indikation zur Splenektomie abgehandelt. Der Hämatologe interessiert sich besonders für die Definition von Hypersplenismus und Dysplenismus. Im ersten Fall wird die Zytopenie durch mechanische oder immunologische Zellzerstörung einerseits, aber auch durch Ausschwemmungs- und Reifungshemmung andererseits, erklärt. Der Dysplenismus, hämatologisch erkennbar an dem Auftreten von Jolly-Körpern, wirkt sich durch Reifungs- und Zellbildungsstörung aus. Die Milzexstirpation kann sowohl bei Hyper- als auch bei Dysplenismus angezeigt sein. Die Monographie ist breit angelegt und enthält über 4000 Literaturstellen. Sie informiert ausführlich über die Auffassungen des Autors und stellt einen interessanten Beitrag zur Milzproblematik dar.

H. Begemann, *Berlin*

Necrologia

Ludwig Heilmeyer

Prof. Ludwig Heilmeyer died unexpectedly on September 6, 1969 at the age of 71, while on vacation in Italy.

Our journal has lost one of its co-founders and member of the Editorial Board. Heilmeyer's important contributions to Haematology will be reviewed in obituary in one of the next issues.

Index rerum ad Vol 41

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